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# THE EFFECT OF ECDYSTEROIDS ON SALIVARY GLAND DEGENERATION AND VITELLOGENESIS IN THE IXODID TICK, *Amblyomma americanum*

by

(C) PAUL JEFFREY LINDSAY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PHYSIOLOGY

DEPARTMENT of ZOOLOGY

EDMONTON, ALBERTA

FALL 1987

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Supervisor

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Date.....May 1/87.....

## Abstract

Female ixodid ticks employ their salivary glands as osmo- and volume regulatory organs. As the female imbibes blood, she secretes a copious volume of dilute fluid back into the host via the salivary glands. After 7-14 days of feeding (depending on the species), the engorged tick drops off the host. During the next few days, the salivary glands degenerate and vitellogenesis is initiated.

The course of salivary gland degeneration was studied in the ixodid tick, *Amblyomma americanum* L.. Salivary glands of ticks weighing >60 mg lost virtually all their secretory competence by 4 days post-removal from the host. Glands from smaller females (20-60 mg) remained competent at 4 and 7 days post-removal (although the rate of transport was approx. 50% less than on day 0). This loss in fluid secretion was not due to autolysis however, because if the ticks were allowed to feed again for 2-3 days, their glands regained full competency.

Salivary glands taken from large partially fed ticks (60-120 mg) and cultured for 4 days, secrete 64% of the rate of glands tested on day 0 post-removal. Hence, cultured glands of large partially fed ticks secrete as well as non-cultured glands from day 4 small partially fed ticks. In the presence of several ecdysteroids (1 µg/ml), however, the cultured salivary glands showed true signs of degeneration.

Interestingly, vertebrate steroids and 2-deoxyecydsone (1 μg/ml) increased fluid secretion compared to control glands. The minimal structural requirements for molecules having ecdysteroid action are similar to those reported for insects. The vertebrate steroids and 2-deoxyecdysone lack these structural requirements.

Vitellogenesis proceeds during the time that salivary gland degeneration occurs. I, therefore, studied whether ecdysteroids could also induce vitellogenesis. None of the ecdysteroids stimulated vitellogenesis *in vitro*; however, substrates and/or the tissues required to synthesize yolk proteins may have been lacking. I also injected female ticks with azadirachtin (1-50 μg/g tick weight), an insect growth regulator which is reputed to block ecdysteroid action in insects. Azadirachtin did not inhibit vitellogenesis nor the number of eggs laid by the ticks. Moreover, azadirachtin also did not attenuate salivary gland degeneration - a proven ecdysteroid-sensitive system. Azadirachtin's ineffectiveness could be explained by the following: 1) Like the salivary gland system, vitellogenesis may be an ecdysteroid-sensitive system which is insensitive to azadirachtin. 2) Ecdysteroids may not be involved in vitellogenesis in ticks.

**Acknowledgements**

The completion of this thesis would not have been possible without the guidance of the following people: my committee members, Dr. W.A. McBlain (Department of Medicine, University of Alberta), Dr. W.M. Samuel and Dr. N.E. Stacey (Department of Zoology, University of Alberta); Dr. B. Gupta (Department of Zoology, Cambridge University) for his advice regarding the preparation of salivary gland tissue for electron microscopy; and my supervisor, Dr. W.R. Kaufman, whose never ending faith in my abilities enabled me to pursue this degree. Of course, two and one-half years of research involves a lot of fun times as well. For this I must thank Isabelle Côté, Stephen Cozens and the entire sixth floor of Zoology, past and present, for the many hours of entertaining coffee breaks and moments of enlightenment. Finally, I want to thank my parents for their support during my entire University career.

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# I. General Introduction

Ticks of the family Ixodidae employ their salivary glands for numerous functions: In most species, the glands secrete a cement, thus preventing dislodgement by the host (Moorhouse, 1969). The salivary glands also secrete a hygroscopic substance as part of a water vapour uptake mechanism when the tick has been dehydrated (Knülle & Devine, 1972), and anti-coagulants to prevent clogging of the food channel (Hellman & Hawkins, 1967). The salivary glands are also the principal organs of osmoregulation in the female (though probably not the male) during the blood meal. It is also via saliva that most of the pathogens transmitted by ixodid ticks gain access to the host. Because of these and other functions (see Sauer, 1977), salivary glands of ticks have attracted much attention among acarologists.

The salivary glands of female ixodid ticks are composed of three types of acini. The type I acini are found in the anterior portion of the gland and drain their secretion directly into the main salivary duct. The type I acinus probably secretes the hygroscopic substance (mentioned above) which allows the unfed tick to gain water from the air (McMullen *et al*. 1976; Needham & Coons, 1984). Type II acini are found mainly at the proximal parts of the secondary ducts (branches of the main salivary duct) and may

contribute to cement production and fluid secretion (Fawcett *et al.*, 1986). Type III acini are located on the distal portions of the lobular ducts (branches of the secondary ducts) and, as well as secreting cement, are the major acini involved in osmo- and volume regulation during feeding (Meredith & Kaufman, 1973; Fawcett *et al.*, 1981).

Ixodid ticks usually take 7-14 days to feed to repletion. Feeding can be divided into two phases: a slow phase lasting most of the feeding period, and a rapid phase which occurs during the last 12-24 hours (Snow, 1969). During the slow phase, *Amblyomma hebraeum* Koch females increase in weight from 30 mg (unfed) to approximately 300-400 mg. To prevent excess dilution of its own body fluids, the tick must dispose of a large volume of fluid from the blood meal. Fluid excretion by ixodid ticks is not accomplished by the Malpighian tubules as in other haematophagous arthropods. It is instead carried out by the salivary glands (Gregson, 1967; Tatchell, 1967; Kaufman & Phillips, 1973). Kaufman (1976) demonstrated that, at the onset of feeding, the salivary glands are incapable of excreting the large volumes of saliva needed for osmoregulation. However, radical cytological changes occur in the cells of the type III acinus during the feeding period (Megaw & Beadle, 1979). Most notably, the f-cell and ablumenal interstitial cell show a marked increase in the number of cell processes which form a labyrinth of interdigitating cell membrane (Fawcett *et al.*, 1981). These

changes enhance the fluid secretory ability of the salivary gland.

When the tick finishes feeding, the salivary glands begin to degenerate (Till, 1961). At this time, autophagic vacuoles appear in certain cells of the type III acini (Harris & Kauman, 1981). Salivary gland autolysis is triggered by a haemolymph borne factor, 'tick salivary gland degeneration factor' (TSGDF, Harris & Kaufman, 1981; 1984). Harris & Kaufman (1985) induced salivary gland degeneration *in vitro* by exposing glands of female *A. hebraeum* to the arthropod moulting hormones, ecdysone (E) and 20-hydroxyecdysone (20-OHE). Similar results were obtained when intact females were infused with 20-OHE for 24 h.

Following drop-off from the host, a number of significant events occur: a) ecdysteroid levels in haemolymph and other tissues increase markedly prior to oviposition (Connat *et al.*, 1985); b) salivary gland degeneration occurs within 3-4 days post-engorgement; c) vitellogenesis occurs within 4-10 days post-engorgement; and d) resorption of the endocuticle occurs throughout this period (Lees, 1952). Because the above work has suggested an important role for ecdysteroids in the female tick post-engorgement, I wished to examine further the action of ecdysteroids on salivary gland degeneration and vitellogenesis. This thesis will explore ecdysteroid involvement in these two events.

## II. Salivary Gland Development and Degeneration in *A. americanum*

### A. Introduction

Degeneration of the salivary glands of ticks was first recorded by Vitzhum (1944; quoted by Till (1961). Till (1961) described the appearance of phagocytic cells amongst the degenerating acini of the salivary glands of female *Ripicephalus appendiculatus* Neumann, within a few days post-engorgement. Degeneration of the salivary glands has also been observed in *A. hebraeum* (Harris & Kaufman, 1981; Connat *et al.*, 1985).

In order to test whether salivary gland degeneration is controlled by a hormone, Harris & Kaufman (1981) implanted salivary glands from partially fed *A. hebraeum* into the haemocoel of engorged females. Two days later, the type III acini of these salivary glands possessed autophagic vacuoles containing cell debris of various organelles. Similar salivary glands transplanted to the haemocoel of small partially fed ticks did not have this ultrastructural feature characteristic of autolysis. These results indicate that a hormone ('tick salivary gland degeneration factor' TSGDF) triggers degeneration of the salivary glands of engorged females.

Recent evidence has suggested that TSGDF is an ecdysteroid (Harris & Kaufman, 1985), a class of steroids related to the arthropod moulting hormone, ecdysone (E). For example, haemolymph titres of ecdysteroid increase about 50 fold in female *A. hebraeum* within 10 days post-engorgement (Connat *et al.*, 1985). Moreover, Harris & Kaufman (1985) demonstrated that exogenous 20-OHE infused over 24 h into partially fed ticks, triggers a dose dependant degeneration of the salivary glands.

I wanted to conduct studies of structure-activity relationships of ecdysteroids and vertebrate steroids on salivary glands in organ culture in order to probe the specificity of the ecdysteroid receptor. Unfortunately, however, the *A. hebraeum* colony died and I was forced to turn to another ixodid species, *A. americanum* L.. Thus, I had to re-establish some of the basic parameters of salivary gland development and degeneration already determined for *A. hebraeum*. These included: 1) secretory competence of the salivary gland throughout the feeding cycle, 2) the time course for salivary gland degeneration post-engorgement, and 3) the critical weight above which TSGDF is released. This chapter will focus on these parameters and compare them to characteristics of salivary gland degeneration in *A. hebraeum*.

## B. Materials and Methods

### 1) Feeding

Ticks were confined to rabbits as described by Kaufman & Phillips (1973). Briefly, a foam rubber corral topped with cotton cloth was glued using an ammoniacal-based latex (Latex Compounding Co., Toronto, Canada) to a shaven area on backs of mature rabbits. Since rabbits develop an immunity following a single exposure to ticks (Bowessidjaou *et al.*, 1977), a given rabbit served as host for only one batch of ticks.

### 2) Ticks

*A. americanum* ticks were reared in our own laboratory from specimens generously provided by Dr. J.R. Sauer, Department of Entomology, Oklahoma State University. Ticks of all developmental stages were stored in darkness, at 26°C and 95% relative humidity (RH).

#### *a) larvae*

Larvae were fed at least four weeks after the eggs had hatched. The larvae fed to engorgement within 10 days and usually moulted into nymphs within 8-10 days.

#### *b) nymphs*

Nymphs were stored for a minimum of 4 weeks after moulting. They fed to engorgement within 8-10 days and

usually moulted to adults within 24 days.

*c) adults*

Newly moulted adults were transferred to clean vials and stored for at least four weeks before being fed. Because copulation is necessary for full engorgement, an equal number of each sex (usually 60) were confined to the backs of rabbits for feeding. The females usually engorged within 11 days.

From this point onward, ticks weighing 20-60 mg will be referred to as 'small partially fed ticks'; 60-120 mg ticks will be called 'large partially fed ticks'; and 'engorged ticks' will refer to those that weigh >300 mg and which have spontaneously detached from the host.

**3) Assay for Secretory Competence of Salivary Glands**

Harris & Kaufman (1984) demonstrated that a simple quantitative assay could be used as an index for salivary gland degeneration. Each tick was glued (cyonacrylate compound; Cardinal Industries) to a strip of adhesive tape applied to the bottom of a small petri dish. The dish was flooded with a modified Hank's balanced saline (see Appendix for composition). The dorsum was removed using a fine razorblade scalpel and the main salivary ducts were ligated with silk thread. (The silk thread (8-0; Davis & Geck) was cut into approximately 2-cm lengths and then separated into 3 finer strands, each of which was used as needed). The

salivary ducts were severed distal to the ligatures and the glands were transferred to fresh TC medium 199 (Gibco; see Appendix for composition) where the glands remained for approximately 15 min. Wet weights of the ligated glands were measured on a Sartorius 2474 microbalance immediately after gentle blotting, the glands were then incubated in TC medium 199 (at room temp., 22°C) containing 10 μM dopamine (DA, Sigma). This concentration elicits a maximal rate of fluid transport (Kaufman, 1976). The incubation medium was stirred constantly. Following incubation (10 min) the glands were blotted and weighed again to determine the net amount of fluid uptake. The degree of fluid secretory competence can be used as an index of salivary gland degeneration (see Harris & Kaufman, 1984).

**4) Experiments**

Experiment 1: The salivary glands of females weighing 110-200 mg were excised soon after the ticks were removed from the host and incubated in 10 μM DA for 5,10,15 or 20 min (fig. 1). The purpose was to establish the period over which fluid uptake was linear.

Experiment 2: I measured the secretory competence of salivary glands throughout the feeding cycle, because in other species, it is known that as feeding progresses the maximum secretory rate increases until a plateau is reached (Kaufman, 1976; Sauer *et al.*, 1979). Female ticks weighing 20-600 mg were removed from the host and salivary fluid

secretion measured as described above using 10 min incubations (fig. 2).

Experiment 3: In order to examine the course of salivary gland degeneration, females >400 mg (most of which were engorged) were removed, weighed and stored at 95% RH, 26°C. The salivary glands were excised from the ticks on day 0,1,2,3 or 4 post-removal from the host and assayed for secretory competence (fig. 3).

Experiment 4: Salivary glands from females weighing >400 mg secreted almost no fluid at 4 days post-removal (fig. 3). In order to determine the critical weight above which salivary gland degeneration would occur, I removed smaller ticks (20-140 mg) from the host and measured the secretory competence of the salivary glands after 4 or 7 days (fig. 4).

Experiment 5: Experiment 4 showed that small partially fed ticks had a lower fluid secretory rate on day 4 than on day 0. Harris & Kaufman (1984) showed a similar loss of secretory competence in *A. hebraeum*. The loss in *A. hebraeum* was not due to autolysis because if allowed to feed again, the salivary glands of these ticks almost completely regained their secretory competence. I therefore repeated the experiment of Harris & Kaufman (1984) using *A. americanum*. After 4 days removal from the host, small partially fed females were given the opportunity to resume feeding. The ticks fed for another couple of days and were removed and weighed. Their glands were assayed for secretory

competence (fig. 5).

**5) Statistics**

The results are reported as mean ± S.E.M. (n). Statistical significance (analysis of variance (ANOVA) and Student's t-tests, as appropriate) were calculated using the MIDAS statistical package of the University of Alberta's main computer (MTS). Statistical significance is indicated either at the $0.01<p<0.05$ (*) or $p<0.01$ (**) level.

## Results

Experiment 1: The time course for fluid uptake was measured for salivary glands of large partially fed ticks within 2 h removal from the host. Fluid transport increased in a linear fashion for the first 15 minutes of incubation (fig. 1). On the basis of these results, a 10 min incubation period was adopted for further experiments in order to ensure linear transport kinetics.

Experiment 2: Fig. 2 shows the secretory competence of salivary glands from females during the whole feeding cycle. Secretory rates rose gradually from that for small partially fed ticks (1.71 ± 0.11 mg/gland/10 min, n=43) to peak in ticks weighing 150-180 mg (3.72 ± 0.29 mg/gland/10 min, n=25). Secretory competence of salivary glands from larger ticks (>360 mg) decreased to 2.18 ± 0.4 mg/gland/10 min (n=14). Because of the difference in secretory ability of salivary glands from ticks of varying weight, this factor had to be taken into account in the design of all subsequent experiments.

Experiment 3: The secretory rate of salivary glands from engorged ticks (>400 mg) decreased markedly ($p<0.01$) as a function of time post-removal from the host (fig. 3). By day 4 post-removal, the salivary glands lost virtually all their fluid secretory competence
(2.70 ± 0.46 mg/gland/10 min, n=10 vs

0.03 ± 0.01 mg/gland/10 min, n=32 on day 4).

Experiment 4: Salivary glands of partially fed ticks showed a decline in fluid uptake at days 4 and 7 post-removal (fig. 4). Salivary glands from small partially fed ticks lost approximately 50% of their secretory competence by day 4 or 7. Salivary glands of large partially fed ticks assayed 4 days post-removal, secreted 0.12 ± 0.05 mg/gland/10 min (n=11) whereas salivary glands of similar day 0 ticks secreted 2.28 ± 0.19 mg/gland/10 min, n=16 (see experiment 2, fig. 2). Thus, it is apparent that only the salivary glands of small partially fed ticks remain competent by days 4 and 7 (fig. 4). This suggests that in *A. americanum*, the critical body weight for TSGDF release is approximately 60-70 mg.

Experiment 5: When small partially fed ticks were left off the host for 4 days and then put back on, they reattached and fed to a much larger size. The salivary glands of these ticks regained full secretory competence (fig. 5). This indicates that the partial loss in fluid secretion from glands of small partially fed ticks (fig. 4) is not due to salivary gland degeneration; a similar result was also seen previously for *A. hebraeum* (Harris & Kaufman, 1984).

## D. Discussion

As indicated in the general introduction, ticks feed in 2 phases: a slow phase lasting 6-10 days (depending on the species) during which the tick increases in size at a slow, steady pace, and a rapid phase occurring in the last 12-24 hours during which the tick rapidly enlarges to a size approximately 100 times its unfed weight (Snow, 1969). Female *A. hebraeum* reach a weight of about 300 mg during the slow phase, whereas *A. americanum* females feed to a weight of approximately 200 mg (Sauer & Essenberg, 1984).

During the slow phase of feeding, there is a gradual increase in competence which is similar to that for *Dermacentor andersoni* (Kaufman, 1976), and indeed *A. americanum* in another study (Sauer *et al.*, 1979). Fluid secretory rates observed by Sauer *et al.* (1979) were virtually identical to those rates which I observed (200-300 nl/min vs. 2-3 mg/10 min, respectively).

By day 4 post-engorgement, the salivary glands of replete *A. americanum* have lost virtually all their secretory competence (fig. 3). Similarly, in *A. hebraeum*, glands from large female ticks degenerated by day 4 (Harris & Kaufman, 1984). However, the critical weight above which degeneration occurs is only 60-70 mg in *A. americanum* (fig. 4), compared to 250-300 mg in *A. hebraeum*. The reason for this difference may be as follows: unfed *A. hebraeum*

weigh approximately 20-35 mg. Thus, salivary gland degeneration seems to be initiated when the tick increases its weight approximately 10-fold. Perhaps putative stretch receptors in the abdomen monitor increases in abdominal size, thus a 10-fold increase may trigger these receptors, consequently triggering TSGDF release. Unfed *A. americanum* weigh only 3-5 mg; therefore, one might expect salivary gland degeneration to occur at a much lower absolute weight in *A. americanum*, since relative stretch of the abdomen would be similar in both cases.

The salivary glands of small *A. hebraeum* females (200-300 mg; i.e. below the critical weight) lose 75% of their secretory ability on day 4 and remain at this level at least up to day 15 post-removal (Harris & Kaufman, 1984). This loss of secretion is not due to degeneration because the glands regain almost their full secretory competence if the ticks are allowed to recommence feeding. Also, ultrastructural examination of salivary glands of these ticks confirmed a dearth of autophagic vacuoles (Harris & Kaufman, 1985). Fig. 5 shows that my results for *A. americanum* are similar. These experiments indicate that the loss of fluid secretory competence alone cannot be used as an index for degeneration. Therefore, only a loss of secretory ability below the level found in small partially fed ticks 4 days post-removal, can be attributed to degeneration of the salivary gland.

In the next chapter, I examine the effect of exogenous ecdysteroids and vertebrate steroids on salivary glands in organ culture.

Fig. 1: Fluid uptake in salivary glands of *A. americanum* ticks (100-200 mg) day 0 post-removal when exposed to 10 μM DA for 5,10,15 or 20 min. Individual salivary glands were ligated, weighed and exposed to DA at one of the time periods indicated. Means ± S.E.M. and n are shown. Fluid transport increased significantly in a linear fashion for 15 min. In this and all other figures, differences were compared using a 1-way ANOVA followed by post-hoc tests, unless otherwise stated.

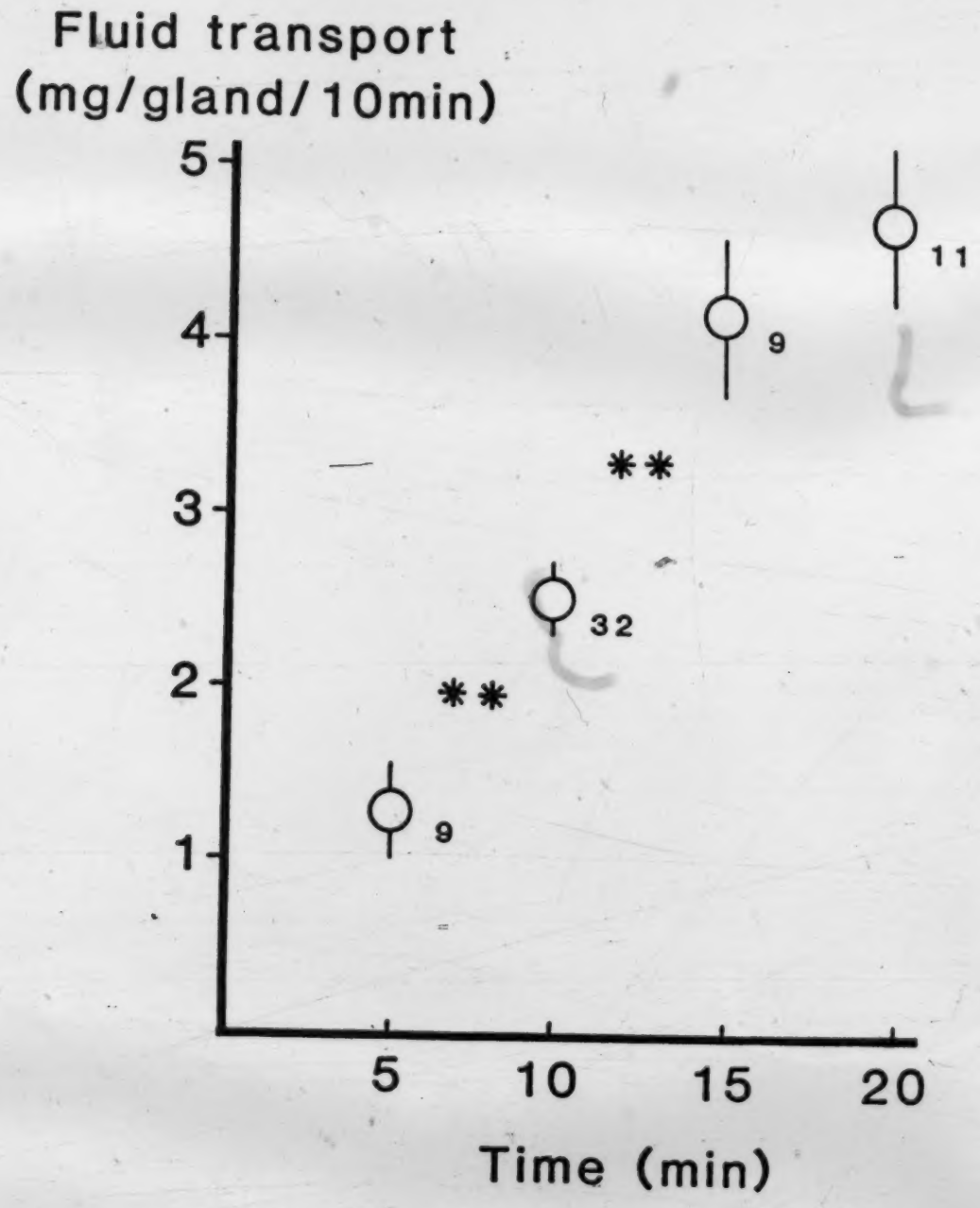

Fluid transport
(mg/gland/10min)
5
4
3
2
1
11
9
**
32
**
9
5
10
15
20
Time (min)

Fig. 2: Effect of 10 μM DA on fluid secretory competence of salivary glands from *A. americanum* throughout the feeding cycle. Means ± S.E.M. and n are shown. Fluid secretion from salivary glands of ticks weighing 120.1-210 mg were significantly higher than <120.1 mg ticks ($0.01<p<0.05$). Fluid secretion from salivary glands of 150.1-180 mg ticks was significantly greater than >210.1 mg ticks ($0.01<p<0.05$).

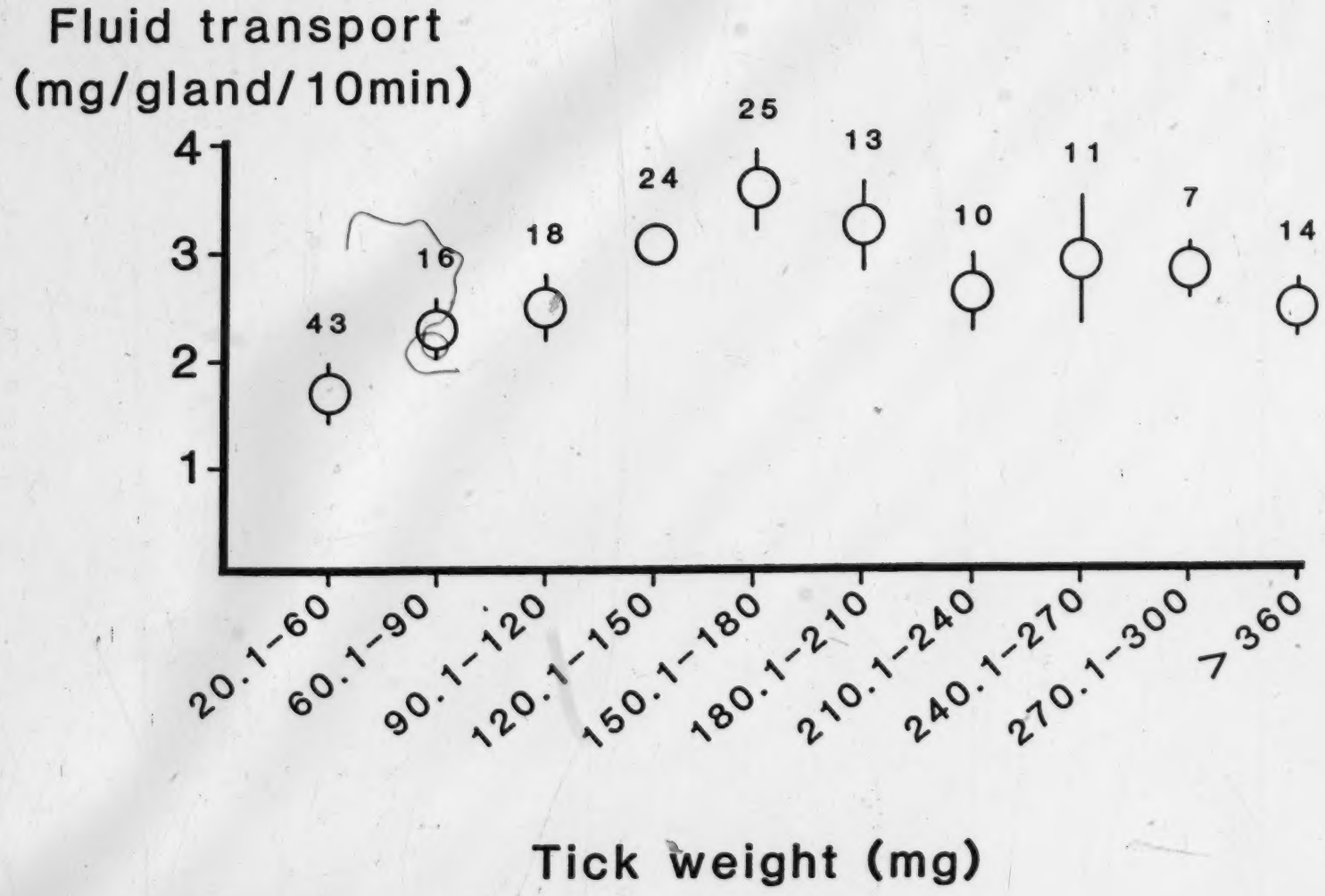

Fluid transport
(mg/gland/10min)
4
3
2
1
43
16
18
24
25
13
10
11
7
14
20.1-60
60.1-90
90.1-120
120.1-150
150.1-180
180.1-210
210.1-240
240.1-270
270.1-300
> 360
Tick weight (mg)

Fig. 3: Reduction of secretory competence of salivary glands from engorged *A. americanum* ticks as a function of time post-removal. By day 3-4, the glands secreted virtually no fluid. Means ± S.E.M. and n are shown. Fluid secretion decreased significantly as days post-removal increased ($p<0.01$).

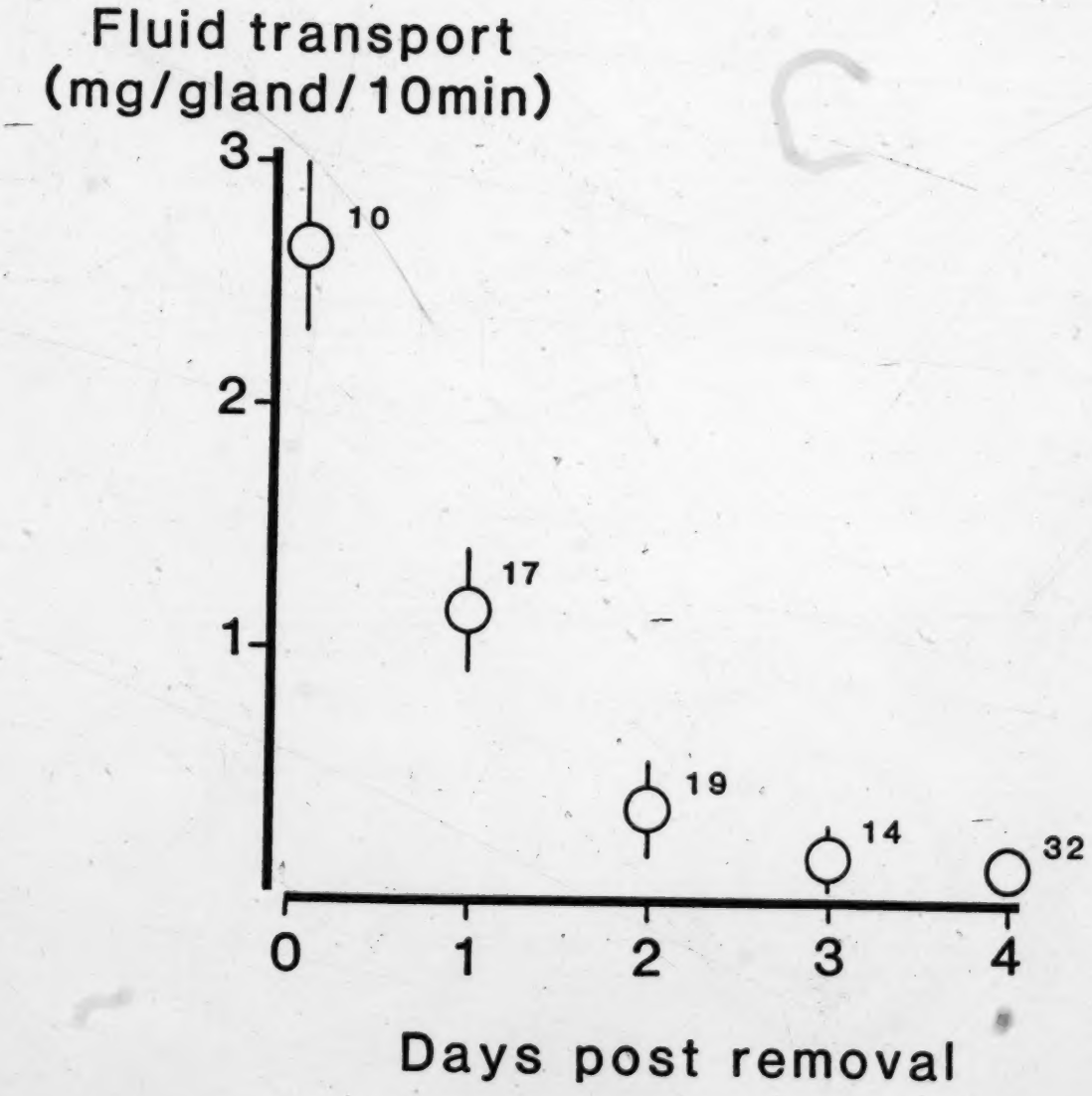

Fluid transport
(mg/gland/10min)
3
2
1
10
17
19
14
32
0
1
2
3
4
Days post removal

Fig. 4: Secretory competence of salivary glands from small partially fed *A. americanum* ticks as a function of time post-removal (Day 0 (◯); Day 4 (△)); Day 7 (□). Means ± S.E.M. and n are shown. Fluid secretion from salivary glands of ticks excised at day 4 and 7 was significantly less than Day 0 ($p<0.01$ for day 4 and 7 ticks weighing <40 mg and >50.1 mg; $0.01<p<0.05$ for day 4 and 7 ticks weighing 40.1-50 mg). There was no significant difference in fluid secretion between small partially fed ticks assayed on day 4 and 7 ($p>0.05$). However, fluid secretion at day 4 was significantly less in >60 mg ticks than in <60 mg ticks ($p<0.01$).

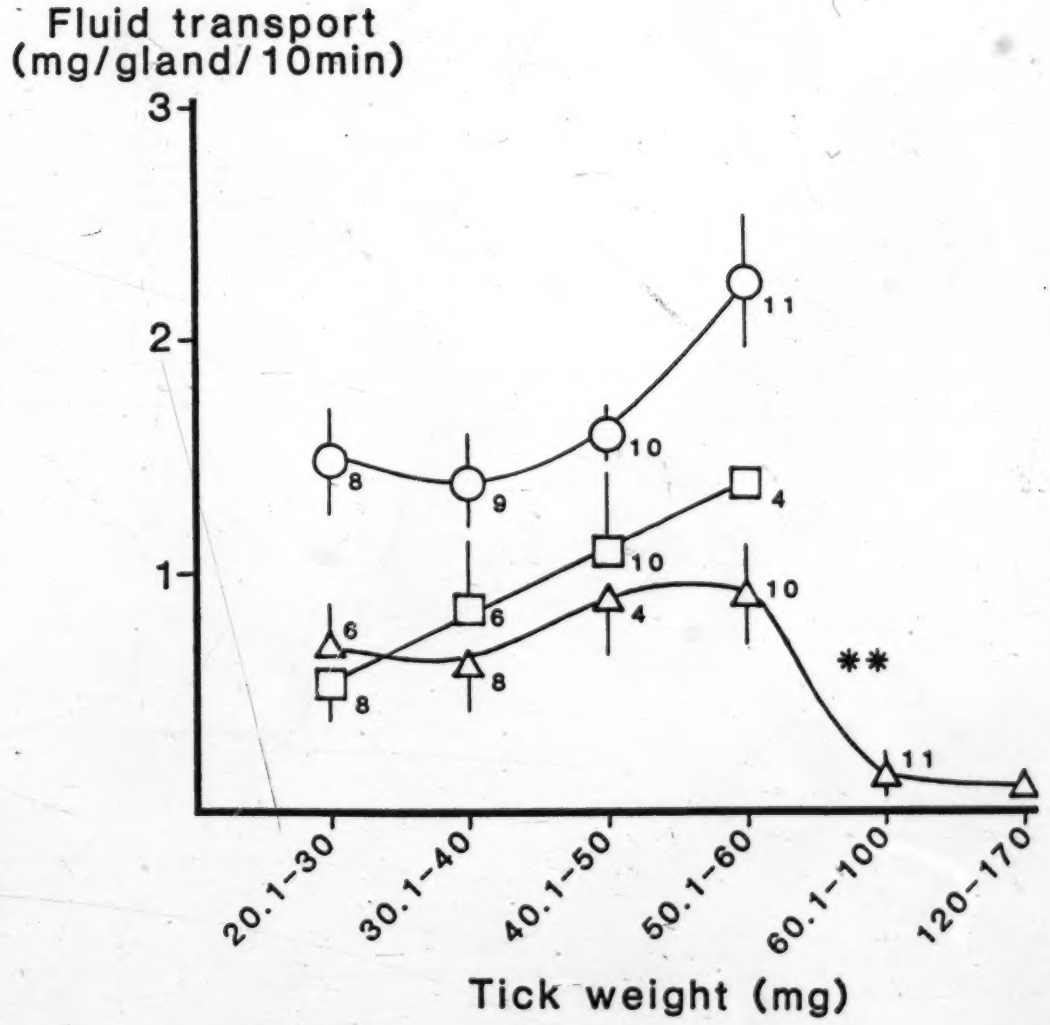
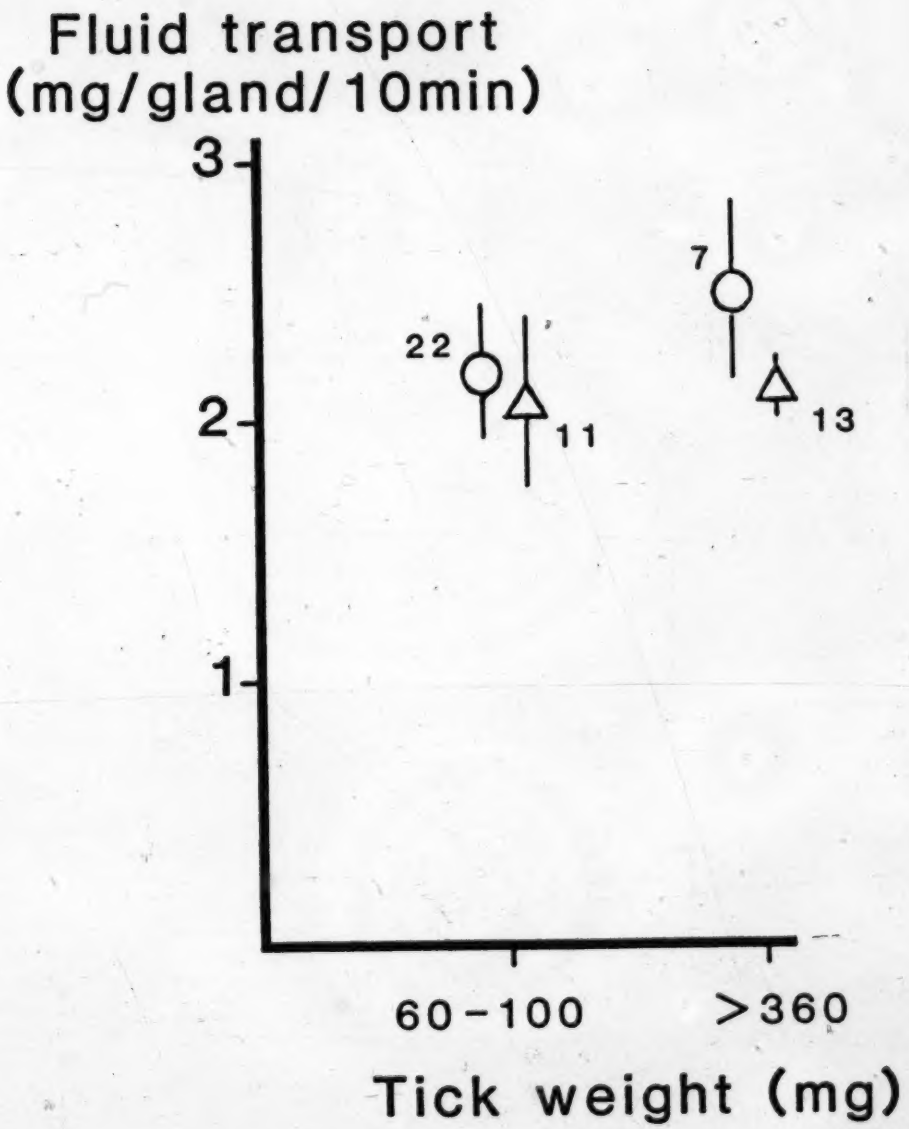

Fluid transport
(mg/gland/10min)
3
2
1
11
10
8
9
4
10
6
6
10
4
8
8
**
11
20.1-30
30.1-40
40.1-50
50.1-60
60.1-100
120-170
Tick weight (mg)

Fig. 5: Restoration of secretory competence of salivary glands from small partially fed *A. americanum* ticks (20-60 mg) which after 4 days post removal were put back on the host to feed. The ticks fed for a day or so (up to 500 mg in some cases), were then removed from the host and their glands were measured for secretory competence (△). Engorged ticks which had fed continuously (i.e. not removed from host at a small weight) had their glands assayed at day 0 post engorgement, for comparison (◯). Means ± S.E.M. and n are shown. Differences (NS) were compared using the Student's t-test.


Fluid transport
(mg/gland/10min)
3
2
1
22
11
7
13
60-100
>360
Tick weight (mg)

## III. Structure-activity Relationships of Ecdysteroids and Vertebrate Steroids on Salivary Gland Degeneration

### A. Introduction

Recent evidence strongly suggests that TSGDF is an ecdysteroid. Harris & Kaufman (1985) induced salivary gland degeneration in *A. hebraeum* after 4 days *in vitro* using E or 20-OHE (30-300 ng/ml). They also showed that infusion of 20-OHE over a 24 h period into small partially fed ticks induces degeneration of the salivary glands. Moreover, 20-OHE and E are effective within the physiological range, as measured by Connat *et al.* (1985) using radioimmunoassay (12 ng/ml in unfed and day 0 post-engorgement females, to 600 ng/ml one day prior to oviposition).

In some insects, ecdysteroids are also involved in vitellogenesis. The best example is the mosquito, *Aedes aegypti*, which requires a blood meal to develop a batch of eggs (Fallon *et al.*, 1974). When unfed females are injected with 20-OHE, viable eggs are produced. Hagedorn *et al.* (1975) demonstrated that 20-OHE activates vitellogenin synthesis in the fat body of this mosquito.

During vitellogenesis there is a rise in haemolymph and ovarian ecdysteroid levels in ticks (Connat *et al.*, 1985). However, ecdysteroids have not yet been shown to stimulate vitellogenesis in ticks. It is possible that the ovarian

ecdysteroids are used for some other function. For example, ecdysteroids also accumulate in the ovary of many insects (Lagueux *et al.*, 1981; Hagedorn, 1983). It is believed that ovarian ecdysteroids accumulate in the eggs and eventually are used to enable the larva to hatch (Lagueux *et al.*, 1979).

In this chapter, I address: 1) the effect of various ecdysteroids and vertebrate steroids on salivary gland degeneration *in vitro*; and 2) the effect of ecdysteroids *in vitro*, on vitellogenesis in the ovary.

## B. Materials and Methods

### 1) Organ culture Assay

I used the 'backless tick explant' organ culture method (Bell, 1980) as modified by Harris & Kaufman (1985). Female ticks were removed from the host, washed in distilled water, weighed and surface sterilized by submersion for 1 min in 1% thimerosol (Sigma) and then 1 min in 70% ethanol (ETOH). The ticks were glued ventral side down to the bottom of a sterile disposable petri dish in a horizontal laminar flow sterile air cabinet. The tick was covered with sterile TC medium 199 (for preparation, see Appendix), the dorsum was removed and the gut was excised and discarded. This 'explant' was rinsed 3 times and then covered with 5 ml sterile medium. The ticks were held at 95% RH, 26°C for 3-5 days and the salivary glands tested for fluid secretory competence. Where applicable, the ovary was assayed for degree of vitellogenesis as described in chapter 4.

### 2) Infusion Experiment

A Harvard microlitre-syringe pump was custom fitted by the company with a two-tier platform bearing slots for 12 syringes. The solution to be infused was taken up in 1 ml all-glass syringes fitted with 30-gauge needles. The pump was run for at least 15-20 min before mounting the ticks in order to achieve a stable rate of fluid delivery. Prior to mounting it on the apparatus, each tick was injected with a

priming dose of the infusion medium (2 µl/100 mg tick body weight), by means of an 'Agla' micrometer syringe (Wellcome Reagents Ltd). To mount the tick, the needle was introduced into the haemocoel through the camerostomal fold (the articulation between the scutum and capitulum). All ticks were infused for 24 h at a rate of 5.64 ± 0.8 µl/h (n=8).

**3) Electron Microscopy**

After the salivary glands were assayed for fluid secretory competence, they were prepared for electron microscopy as described by Fawcett *et al*. (1981) with some modifications. The glands were trimmed, immersed in 2.5% gluteraldehyde in 0.05 M cacodylate buffer, containing 0.15 M sucrose at 10°C, and stirred every 5 min for 30 min. The glands were then post fixed in 1% osmium tetroxide at room temperature for 90 min. After fixation, the tissue was washed for three 5-min periods in Mellonig's phosphate buffer (for composition, see Appendix). After this, the tissue was treated with two more 5-min washes in distilled water. The glands were stained *en bloc* in 0.5% uranyl acetate for 1 h, rinsed for 5 min in distilled water and dehydrated in ETOH (50,70,85,95 and 100%) for 10 min at each concentration. Then the glands were given three 10-min washes in propylene oxide and embedded overnight at room temperature in 25% EPON, 75% propylene oxide. The tissue was transferred to a 50/50 EPON/propylene oxide mixture for 2 h before being finally embedded in 100% EPON and incubated for

48 h at 60°C in a small oven.

Thick and thin sections were cut with a glass knife using a LKB 3 microtome. Thick sections (0.5 µm) were stained with toluidine blue (1%) for examination under the light microscope. Thin sections, with a silver/gold to gold interface, were stained using saturated uranyl acetate (45-60 min) followed by saturated lead citrate (1-1.5 min). Thin sections were viewed using a Phillips 201 electron microscope.

**4) Experiments**

Experiment 1: To test the secretory competence of salivary glands *in vitro*, backless tick explants were prepared from female ticks weighing 20 mg or more. After 4 days, the salivary glands were tested for fluid secretory competence (fig. 6).

Experiment 2: The highest rate of secretion occurred in ticks weighing 60-120 mg (experiment 1, fig. 6). Therefore, I used ticks in this weight range to study the effect of ecdysteroids and vertebrate steroids on salivary gland degeneration and, in some instances, on vitellogenesis. In preliminary experiments, I found that 4 days in culture was the best compromise for allowing sufficient time for the steroids to act (a culture period of 3 days was too short) and not losing too many cultures due to deterioration or contamination (a 5 day culture was sometimes too long). I tested the following ecdysteroids (figs. 7,8): E, 20-OHE

(Simes, Italy); ponasterone A, ponasterone C, muristerone A, polypodine B, cyasterone (gifts from Dr. K. Nakanishi, Department of Chemistry, Columbia University); and 2-deoxyecdysone (gift from Dr. D.S. Horn, Div. Appl. Organic Chem., CSIRO, Melbourne, Australia). The vertebrate steroids used were: progesterone, testosterone, cortisol, β-estradiol (Sigma; figs. 9,10). All compounds (1 mg/ml) were dissolved in 70% ETOH and diluted in TC 199 to working concentration such that salivary glands were exposed to 1 µg steroid/ml in 0.07% ETOH. Control salivary glands were exposed to 0.07% ETOH.

Experiment 3: Small partially fed *A. hebraeum* females (≤200 mg) were set up for the infusion as described above. The 20-OHE (10 µg/ml) was dissolved in 70% ETOH and diluted such that each tick received 20 µM 20-OHE in 0.07% ETOH. Controls received 0.07% ETOH. All ticks were infused for about 24 h. The females were then moved to an incubator (95% RH, 26°C) and held for 3 days, when the salivary glands were excised and assayed for secretory competence.

C. Results

Experiment 1: Salivary glands from large partially fed ticks (60-120 mg) cultured for 4 days secreted 1.44 ± 0.1 mg/gland/10 min (n=46, fig.6). This was a significant increase over small partially fed ticks (20.1-60 mg) whose cultured glands secreted 0.81 ± 0.08 mg/gland/10 min (n=17, $p<0.01$). Ticks >120 mg had progressively less secretory competence, and ticks >200 mg lost almost all their fluid secretory competence (0.16 ± 0.05 mg/gland/10 min, n=12).

Experiment 2: Most of the ecdysteroids caused a significant decrease in wet weight of the salivary glands after 4 days in organ culture (fig. 7) with 20-OHE having the greatest effect (a 42% decrease in wet weight compared to 0.07% ETOH controls, $p<0.01$; fig. 7). The extent to which the weight loss represented extracellular water loss as opposed to the loss of metabolically active tissue is not known.

Fluid secretion was significantly reduced by all the ecdysteroids except for 2-deoxyecdysone (see below). Significance levels were $p<0.01$ for all the ecdysteroids when compared to ETOH controls (fig. 8). In 20-OHE, glands secreted at only 36% the rate of the ETOH controls. By contrast, 2-deoxyecdysone actually increased salivary gland fluid secretion by 43.3% ($p<0.01$) compared to ETOH controls.

ETOH (0.07%) showed no significant effect on wet weights nor fluid secretion compared with glands cultured in TC medium 199 alone ($p>0.05$; figs. 7 & 8). Harris & Kaufman (1985) observed, however, that 0.07% ETOH enhanced fluid secretion of cultured *A. hebraeum* salivary glands by 61.5% compared to glands cultured without ETOH. I have no explanation for the discrepancy between their observation and mine.

Salivary gland wet weights were not significantly affected by progesterone, testosterone or $\beta$-estradiol ($p>0.05$) after 4 days in culture. However, cortisol caused about a 25% decrease in salivary gland wet weight ($0.01<p<0.05$; fig. 9).

After 4 days in culture, salivary gland secretory competence was significantly increased by all the vertebrate steroids (fig. 10). Progesterone and $\beta$-estradiol, the most effective, enhanced fluid secretion by 53.0 ± 7.7% (n=10) and 50.4 ± 9.4% (n=10; $p<0.01$), respectively.

Ecdysteroid-induced degeneration of the salivary glands was confirmed by ultrastructural observation. Autophagic vacuoles were abundant in certain cells of the type III acinus of salivary glands cultured in 20-OHE (1 µg/ml, fig. 11b). ETOH-treated control salivary glands did not possess many autophagic vacuoles (fig. 11a).

Experiment 3: After 4 days in culture, salivary glands exposed to 20-OHE or E did not lose their fluid secretory competence to the same extent as was observed by Harris & Kaufman (1985) for *A. hebraeum* salivary glands. I thought

this might have been due to one of three reasons: 1) our samples of E and 20-OHE (powder) were several years old and might have deteriorated , 2) *A. americanum* ticks might require a higher concentration of E to achieve complete degeneration or, 3) some other factor necessary for salivary gland degeneration was present in the cultures of Harris & Kaufman's (1985) ticks, but absent from mine. Therefore, 1) I assayed 5 explants in 1 µg/ml 20-OHE prepared from newly purchased hormone. After 4 days, these salivary glands secreted 0.79 ± 0.1 mg/gland/10 min (n=5). The older batch of 20-OHE caused the salivary glands to secrete 0.60 ± 0.08 mg/gland/10 min (n=12). 2) I then tested some explants with fresh 10 µg/ml E or 20-OHE. E-treated salivary glands secreted 0.89 ± 0.07 mg/gland/10 min (n=6), and 20-OHE treated glands secreted 0.72 ± 0.06 mg/gland/10 min (n=9). Thus, a higher concentration of ecdysteroid (10 µg/ml) did not significantly increase the degree of degeneration to more than that induced by 1 µg/ml E or 20-OHE ($p>0.05$). 3) I also tested some *A. hebraeum* females using 1 µg/ml fresh 20-OHE or E and observed similar results as for *A. americanum*, namely a 65% and 70% decrease in fluid secretion from E and 20-OHE-treated glands. 4) Finally, I infused some *A. hebraeum* females with 20 µg/ml 20-OHE and observed more degeneration than was observed for cultured *A. hebraeum* glands. Fluid secretion was 25.4% the rate for salivary glands infused with 0.07% ETOH alone (0.71 ± 0.13 mg/gland/10 min, n=13 vs.

2.63 ± 0.48 mg/gland/10 min, n=9, respectively, $p<0.01$). Thus, some factor may have been absent in my explants which would enable complete salivary gland degeneration to proceed as Harris & Kaufman (1985) demonstrated in *A. hebraeum*.

None of the ecdysteroids stimulated vitellogenesis in the ovaries of the cultured ticks mentioned above. By day 3 in organ culture, ovary homogenates of ETOH control ticks gave an absorbance reading of 6.8 ± 1.1/g ovary weight (n=4), whereas ecdysteroid treated ovaries had an absorbance of only 4.9 ± 0.4/g ovary (n=10; $p>0.05$). I have less data for 4 day organ cultures, but again, ecdysteroids did not appear to initiate vitellogenesis.

## D. Discussion

Harris & Kaufman (1985) showed that salivary glands of small partially fed *A. hebraeum* cultured for 4 days secreted as well as glands of similar ticks which were left untreated for 4 days. Similarly, I found that salivary glands of small partially fed *A. americanum* females cultured for 4 days secreted as well as glands from untreated day 4 small ticks (0.8 ± 0.08 mg/gland/10 min, n=17 for cultured glands vs. 0.77 ± 0.06 mg/gland/10 min, n=28 for normal glands; $p>0.05$). Harris & Kaufman (1985) also demonstrated that salivary glands from large *A. hebraeum* (>400 mg) set up in organ culture within 24 h post removal secreted as well as glands from small (300 mg) ticks cultured for 4 days. Unlike *A. hebraeum*, salivary glands from large (>150 mg) *A. americanum* lost their secretory competence when cultured for 4 days (fig. 6). Hence, it is possible that the factors which trigger TSGDF release in *A. americanum* are 'turned on' earlier than in *A. hebraeum*. Once TSGDF is released and taken up by the tissue, degeneration of the salivary glands cannot be halted by washing TSGDF away in culture (Harris & Kaufman, 1985).

The 20-OHE-induced salivary gland degeneration in *A. americanum* was not as complete as Harris & Kaufman (1985) observed in cultured glands of *A. hebraeum*. Using $^3$[H]-E, Wigglesworth *et al.* (1985) showed that several tick tissues

including Malpighian tubules, ovaries and fat body metabolized E *in vitro* into inactive polar products. In my preparations, only the guts (and probably some of the fat body) were removed. Perhaps the ecdysteroid was inactivated by the remaining tissues. I think the latter unlikely, however, because of the large volume of culture medium (5 ml) and large amount of ecdysteroid used, compared to the size of the tick (60-120 mg).

Fig. 11b shows that 20-OHE specifically induces autophagic activity in the salivary glands.[1] Ecdysteroids are known to mediate autophagocytosis in insect tissues also. After 2 days in organ culture with 20-OHE, fat bodies of *Calpodes* larvae showed abundant autophagic activity (Dean, 1978). Similarly, injection of 5 µg/g 20-OHE induced autophagocytosis in the fat body of last instar larval *Mamestra brassicae* (Sass *et al.*, 1983). High concentrations of E also induce acid phosphatase activity (a marker enzyme for lysosomal activity) in salivary glands of *Drosophila melanogaster* larvae (Aizenzon *et al.*, 1975). Acid phosphatase activity is also much elevated in degenerated salivary glands of *A. hebraeum* (Harris & Kaufman, 1981).

As indicated earlier, ecdysteroids did not induce vitellogenesis in organ culture. Vitellogenin, the precursor for yolk proteins, is manufactured from haem products of the blood meal. Breakdown products, specifically

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[1]It must be realized that fig. 11 shows tissue that was cultured for 4 days; hence the quality of the micrographs does not compare favourably with micrographs of fresh tissue (see Harris & Kaufman, 1981)

haemo-glyco-lipoproteins, are translocated to the haemolymph. These proteins are then incorporated into the fat body and synthesized into vitellogenin (Diehl *et al.*, 1982). For my experiments, organ culture preparation involved removal of haemolymph, guts and some fat body. Since haem derivatives are not present in TC 199, and since some potentially necessary organs for vitellin synthesis were removed, these factors may be responsible for the lack of accumulation of yolk in the ovary in these experiments. Further discussion of this topic will be delayed until chapter 4.

The 20-OHE caused the greatest degree of salivary gland degeneration compared to ponasterone C, muristerone A ($p<0.01$), E ($0.01<p<0.05$), and possibly ponasterone A and cyasterone ($p<0.07$). Ecdysteroids are derived from cholesterol (see fig. 12) and fig. 13 shows the structure of all the ecdysteroids used in this study. Structure-activity relationship studies in insects have shown that the following characteristics are essential for ecdysteroid activity *in vitro* and *in vivo*: 1) a *cis*-fused A/B ring conformation, 2) a 6-keto-7-ene grouping in the B ring, 3) a full sterol side chain (Horn & Bergamasco, 1985), and 4) a 14α-OH group which increases ecdysteroid activity *in vitro*. Except for 2-deoxyecdysone, all the ecdysteroids I tried have the four characteristics mentioned above. Singh *et al.* (1982) observed, using ligated housefly larvae, that 5β-OH ecdysteroids are less active *in vitro* than 5β-H

substituents. Ponasterone C and muristerone A have 5β-OH groups and both were the less effective on tick salivary glands (fig. 8). In my system, polypodine B (having a 5β-OH group also), was just as effective as 20-OHE ($p>0.05$) whereas Singh *et al.* (1982) observed its activity to be 42% that of 20-OHE *in vitro*.

The only ecdysteroid which did not cause salivary gland degeneration was 2-deoxyecdysone. An examination of its A/B ring configuration might explain this. The A/B ring of ecdysteroids exists as 2 isomers: 5α and 5β conformations (fig. 14). In the 5α form, a strong axial-axial steric interaction exists between the 2β-OH and 19-$CH_3$ groups. This interaction destabilizes the molecule; thus the 5β conformation predominates (Horn & Bergamasco, 1985). In deoxyecdysteroids (eg. 2-deoxyecdysone), the 2β-OH/19-$CH_3$ interaction is absent, therefore, the 5α isomer predominates. With a 5α A/B ring, 2-deoxyecdysone is planar. The other ecdysteroids, having a 5β A/B are non-planar molecules (Bergamasco & Horn, 1980). As a result, 2-deoxyecdysone interacts with the ecdysteroid receptor in a different manner from the other ecdysteroids. The action of 2-deoxyecdysone was in fact, very similar to vertebrate steroid action on the salivary glands (fig. 10). Note the molecular structure of 2-dexoyecdysone and the vertebrate steroid is comparable (fig. 15). Indeed, the shape of the A/B ring of 2-deoxyecdysone is very much like the A/B ring of vertebrate steroids. With the absence of the

C2-substituent, vertebrate steroids adopt an A/B ring conformation similar to 2-deoxyecdysone. Also, being planar molecules, the A/B ring of 2-deoxyecdysone and the vertebrate steroids is *trans*-fused (Solomons, 1984). Because the ecdysteroid receptor binds mainly to the $\beta$-face of the ecdysteroid molecule in the region of the A/B ring (Horn & Bergamasco, 1985), this might explain why 2-deoxyecdysone and the vertebrate steroids act differently from the other ecdysteroids.

All the vertebrate steroids significantly increased fluid secretion in cultured salivary glands. Testosterone increases skeletal mass in rats by increasing amino acid incorporation hence promoting tissue growth (Mayer & Rosen, 1975). After 4 days in culture, cortisol also increases fluid secretion in completely isolated cultured salivary glands of *D. andersoni* (Kaufman & Barnett, 1977) and similar glands of *A. hebraeum* (Kaufman, pers. communication). How might these vertebrate steroids improve the state of the salivary gland tissue? Glucocorticoids, which inhibit inflammatory reactions, may do so by stabilizing lysosomal membranes; thus the secretion of hydrolytic enzymes which normally occurs during inflammation is inhibited (Hadley, 1984). Zurier & Weissmann (1973) have proposed a mechanism for this stabilizing effect. Steroid hormone analogues can insert their acyl chains into the hydrophobic layer of the lysosome membrane. Thus the bilayer condenses and restricts the mobility of the acyl chains; hence membrane stability

results. The vertebrate steroids may be acting in a similar manner on the lysosomes in tick salivary glands. As a result, secretory competence of these glands would be greater than salivary glands cultured without the steroid. The membrane stabilization theory is, however, not entirely satisfactory, for the following reasons (Haynes, 1974): 1) A very high concentration of the glucocorticoid ($10^{-4}$M) is needed to observe only minimal anti-inflammatory effects *in vitro*. This concentration is 100-fold greater than that needed to suppress inflammation *in vivo*. 2) A single dose of the steroid is ineffective in stabilizing lysosomes. 3) It is critical for the molecule to have an 11$\beta$- and/or 17$\alpha$-OH group and a 20-keto group to elicit anti-inflammatory action. Progesterone, testosterone and $\beta$-estradiol are without the 11$\beta$-OH and 17$\alpha$-OH substituent, and therefore probably do not act in the same manner as cortisol. Indeed, progesterone and testosterone labilize liver lysosomes *in vitro* (Weissmann, 1969). 4) The glucocorticoid is probably acting more directly on the genome and the observed lysosome stability could just be a secondary effect of the steroid.

Despite the weaknesses of the theory, cortisol and the other vertebrate steroids do increase fluid secretion in tick salivary glands. Progesterone, testosterone and cortisol all have the ability to adopt identical A ring conformations, thus they can act upon the same receptor (Duax *et al.*, 1975).

The '2-step' mechanism for steroid action was first proposed independantly by Jensen *et al.* (1968) and Gorski *et al.* (1968). In the first step, the steroid enters the cell by diffusion and binds to a cytoplasmic receptor. During the second step, the steroid-receptor complex translocates to the nucleus where it induces transcription of specific genes. Thus, the effects of steroids are mediated through mRNA and protein synthesis (fig. 16). Whether this mechanism of action applies to all vertebrate steroids is a matter of recent controversy. Strong evidence now demonstrates that unoccupied estrogen and progesterone (but not glucocorticoid) receptors reside in the nucleus (Walters, 1985). Therefore, instead of the cytoplasmic steroid-receptor complex moving into the nucleus, the steroid appears to bind to a resident nuclear receptor.

The mechanism of action of ecdysteroids is analogous to Jensen *et al.* and Gorski *et al.*'s model for vertebrate steroids (O'Connor, 1985). Using *Chironomus* salivary glands, Clever & Karlson (1960) were the first to show that a direct correlation exists between rising ecdysteroid titres and transcriptional activity (indicated as puffs) in the chromosomes. These puffs result from an accumulation of RNA and proteins (Ashburner *et al.*, 1974).

Ecdysteroids are transported to their target tissues by haemolymph binding proteins (Feyereisen, 1980). Once it reaches the target cell, the ecdysteroid diffuses across the membrane (O'Connor, 1985). It binds to a cytoplasmic

receptor and the hormone-receptor complex is translocated to the nucleus where the hormone disengages from the cytoplasmic receptor and binds to the nuclear receptor (Yund *et al.*, 1978). The hormone-nuclear receptor complex then binds to specific genes to modulate transcriptional activity. One model for ecdysteroid action is based on the following data: 1) Ashburner *et al* (1974) observed that two intermolt puffs (i.e. puffs present before ecdysteroid application) regress, and early (within 5-10 min) puffs and late (at least 3 h) puffs appear upon 20-OHE exposure. 2) Cyclohexamide, a protein synthesis inhibitor, does not affect induction of early puffs, but it does inhibit both the normal repression of the early puffs and the appearance of late puffs. 3) Premature removal of the hormone leads to a regression of the early puffs and premature induction of late puffs. The central proposition of the model is a double control upon both the early and late puff sites (fig. 17). The ecdysteroid-receptor (E-R) complex has a positive effect upon the early site causing an induction of mRNA synthesis and ultimately new protein synthesis. At the same time, the E-R complex acts to inhibit RNA synthesis at late puff sites. Only when a sufficient concentration of the protein encoded by the early loci has been synthesized can the E-R complex be displaced from the late site and transcription at that site occur. Displacement of the E-R complex will simultaneously cause regression of the early puffs.

There are a number of similarities in steroid action between vertebrates and insects. In both cases, the hormone enters the cell and becomes bound in the nucleus in a process mediated by a receptor protein. At the time when the first hormonal effects on protein synthesis are observed, at least 90% of the total binding for each steroid type is tightly associated in the nucleus (Yund, 1980). Also, the number of nuclear binding sites per unit DNA is similar for the two classes of steroids. There are also a few differences in steroid hormone action between insects and vertebrates. The most striking of these is the fact that insect cell lines which have not previously been exposed to the steroid hormone (i.e. näive) contain a resident population of nuclear receptors for the insect steroid (Yund, 1980). Controversy exists over the absence of resident nuclear receptors in näive vertebrate target cells. Therefore, at least with glucocorticoids, once the vertebrate steroid binds to the receptor, the complex enters the nucleus to bind to the chromosome.

Ultrastructural evidence clearly indicates that ecdysteroids cause degeneration of tick salivary glands *in vitro* (fig. 11). Structure-activity relationship studies show that 20-OHE elicits the greatest degree of salivary gland degeneration. Vertebrate steroids, having a conformational structure different from ecdysteroids, increase salivary gland fluid secretion. Because salivary glands secrete well in organ culture, this simple bioassay

allows for the determination of the specificity of the putative ecdysteroid receptor.

Fig. 6: Comparison of the secretory competence of salivary glands from *A. americanum* ticks removed from the host and assayed on day 0 (◯), with glands from ticks set up in organ culture (95% rh, 26°C) for 4 days (⬡). Means ± S.E.M. and n are shown. After 4 days in culture, glands of ticks weighing up to 120 mg secreted 64% of the rate of glands tested on day 0 ($p<0.01$). Almost complete salivary gland degeneration was observed in ticks >200 mg. Data for day 0 were taken from fig. 2. Differences were compared using the Student's t-test.

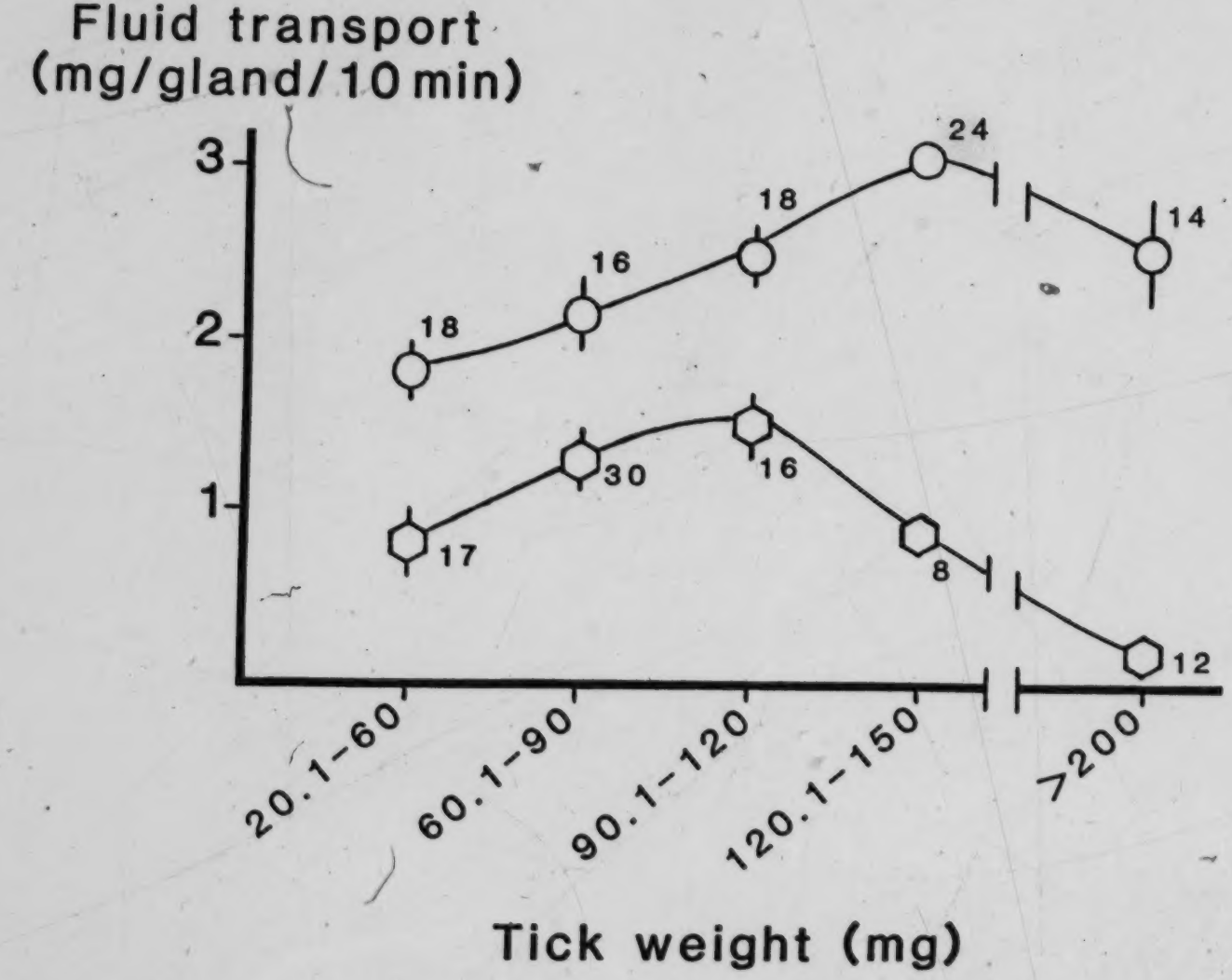

Fluid transport
(mg/gland/10 min)
3
2
1
24
18
16
18
14
18
30
16
17
8
12
20.1-60
60.1-90
90.1-120
120.1-150
>200
Tick weight (mg)

Fig. 7: Effect of ecdysteroids (1 µg/ml) on wet weights of salivary glands of *A. americanum* ticks (60-120 mg) set up in organ culture for 4 days. Except for 2-deoxyecdysone and ponasterone A, the ecdysteroids caused a significant decrease in salivary gland wet weights ($p<0.01$). Means ± S.E.M., and n are shown. For the sake of comparison, salivary glands cultured for 4 days in TC medium 199 without ETOH (no ETOH) are included.

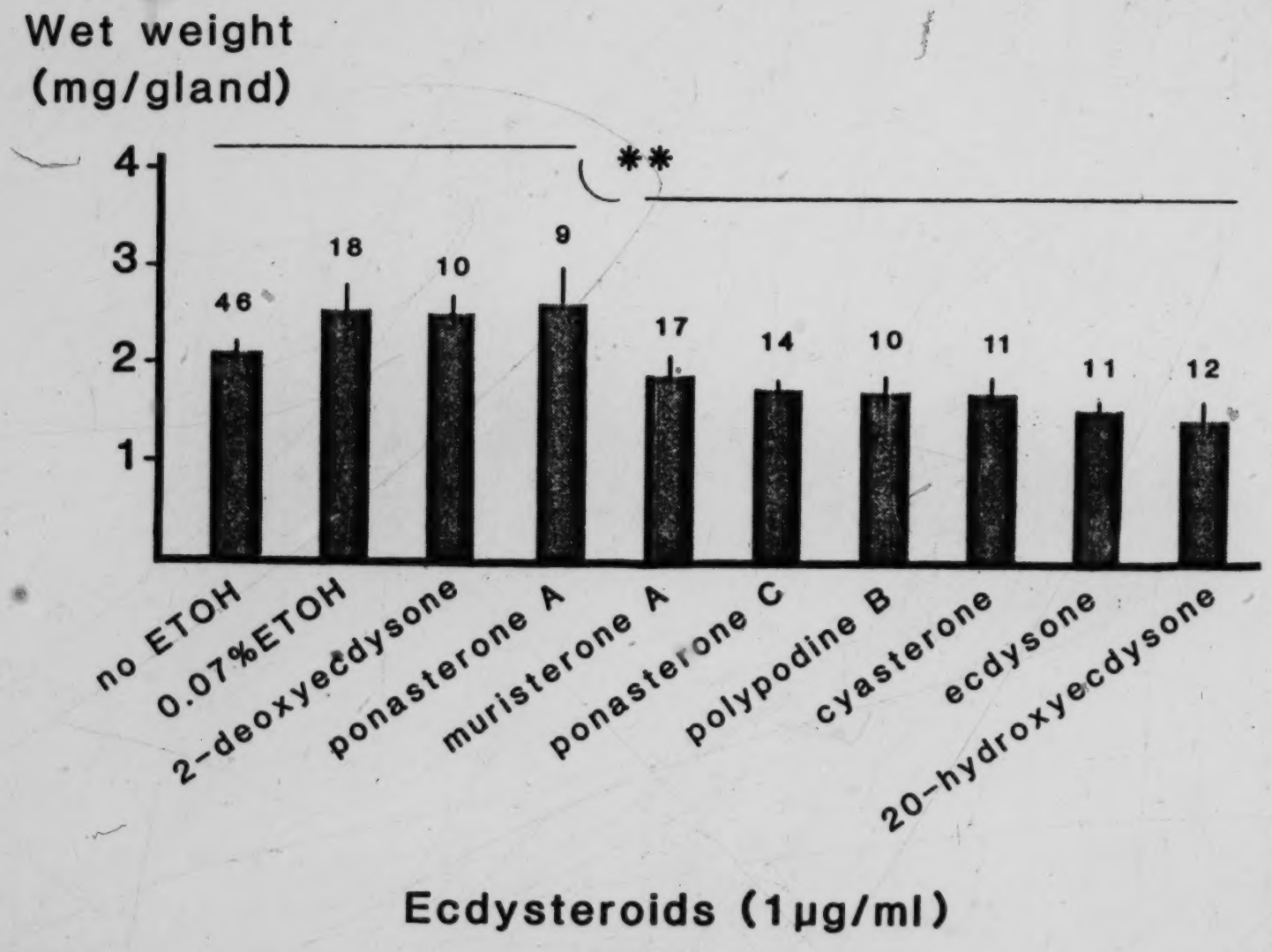

Wet weight
(mg/gland)
4
3
2
1
**
46
18
10
9
17
14
10
11
11
12
no ETOH
0.07%ETOH
2-deoxyecdysone
ponasterone A
muristerone A
ponasterone C
polypodine B
cyasterone
ecdysone
20-hydroxyecdysone
Ecdysteroids (1µg/ml)

Fig. 8: Effect of ecdysteroids (1 µg/ml) on fluid secretion of salivary glands of *A. americanum* ticks (60-120 mg) set up in organ culture for 4 days. Means ± S.E.M., and n are shown. With the exception of 2-deoxyecdysone, all the ecdysteroids significantly decreased fluid secretion in the salivary glands. For the sake of comparison, salivary glands cultured for 4 days in TC 199 without ETOH (No ETOH) are included.

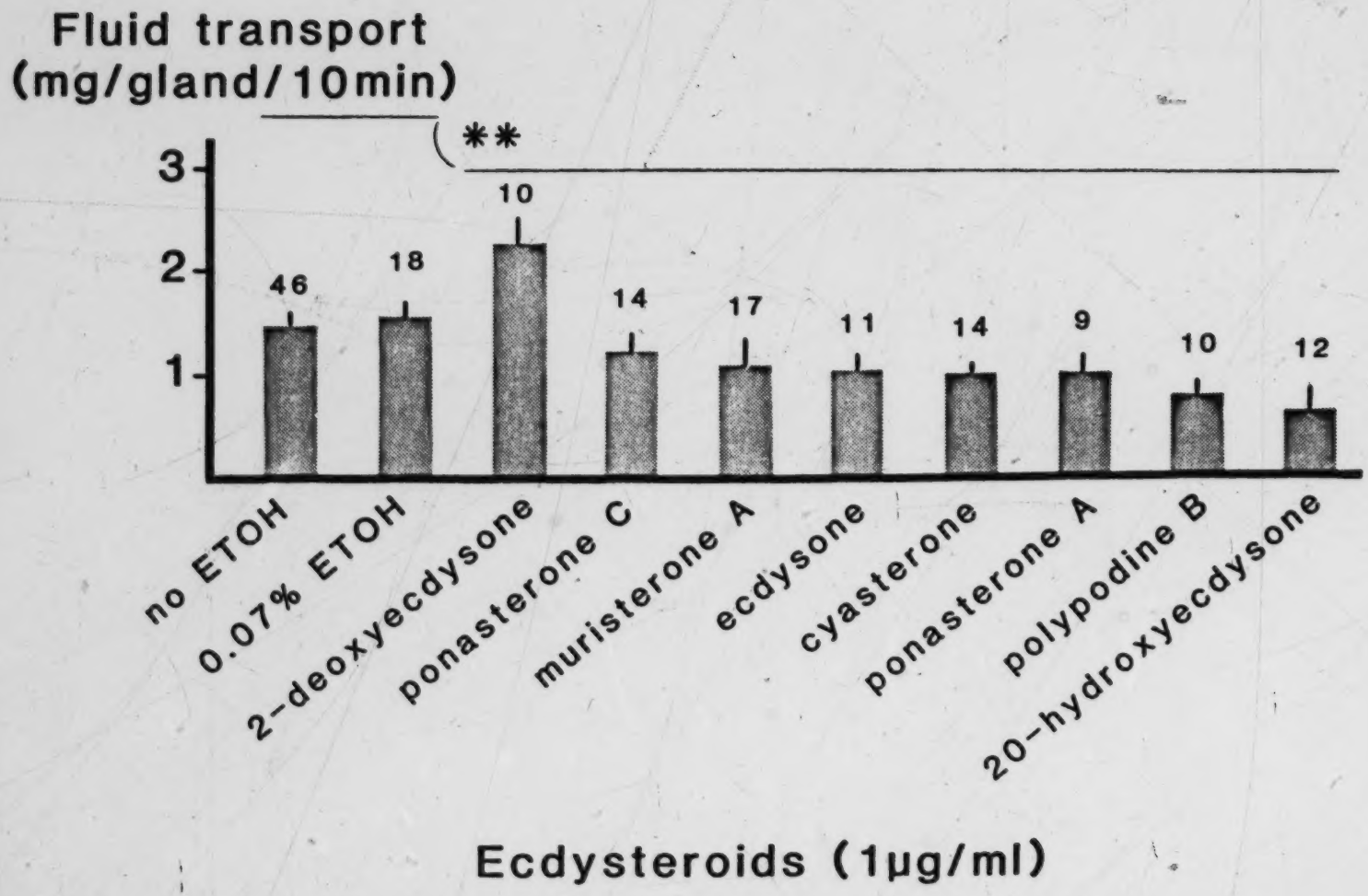

Fluid transport
(mg/gland/10min)
**
3
2
1
46
18
10
14
17
11
14
9
10
12
no ETOH
0.07% ETOH
2-deoxyecdysone
ponasterone C
muristerone A
ecdysone
cyasterone
ponasterone A
polypodine B
20-hydroxyecdysone
Ecdysteroids (1µg/ml)

Fig. 9: Effect of steroids (1 μg/ml) on wet weights of salivary glands of *A. americanum* ticks (60-120 mg) set up in organ culture for 4 days. Means ± S.E.M., and n are shown. Salivary gland wet weights were not affected by testosterone, progesterone nor β-estradiol ($p>0.05$). Only cortisol caused a significant reduction in wet weight ($0.01<p<0.05$). Data for "no ETOH" were copied from fig. 7.

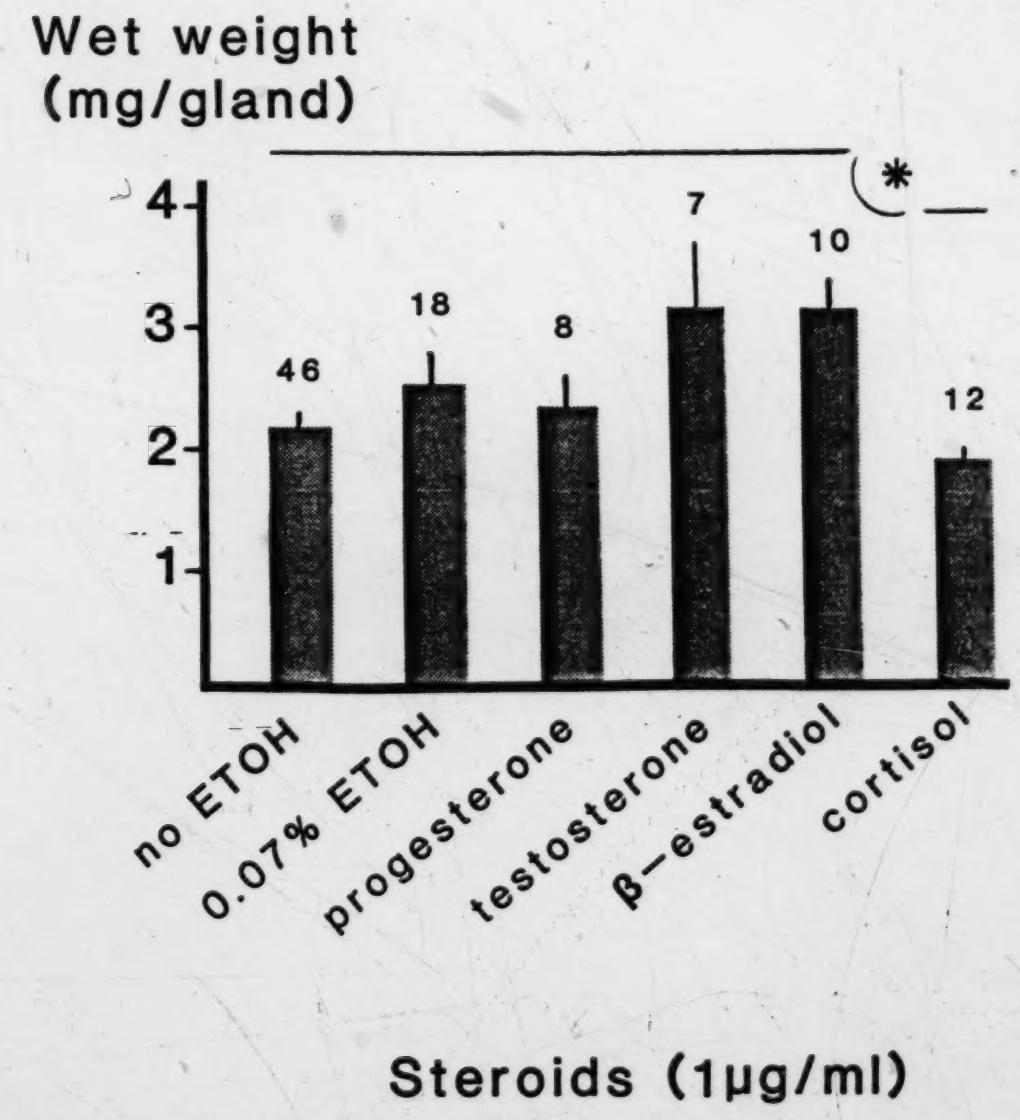

Wet weight
(mg/gland)
4
3
2
1
46
18
8
7
10
12
*
no ETOH
0.07% ETOH
progesterone
testosterone
β-estradiol
cortisol
Steroids (1µg/ml)

Fig. 10: Effect of steroids (1 µg/ml) on fluid secretion of salivary glands of *A. americanum* ticks (60-120 mg) set up in organ culture for 4 days. Means ± S.E.M., and n are shown. In all cases, the vertebrate steroids increased fluid secretion in these salivary glands. Data for "No ETOH" were copied from fig. 8.

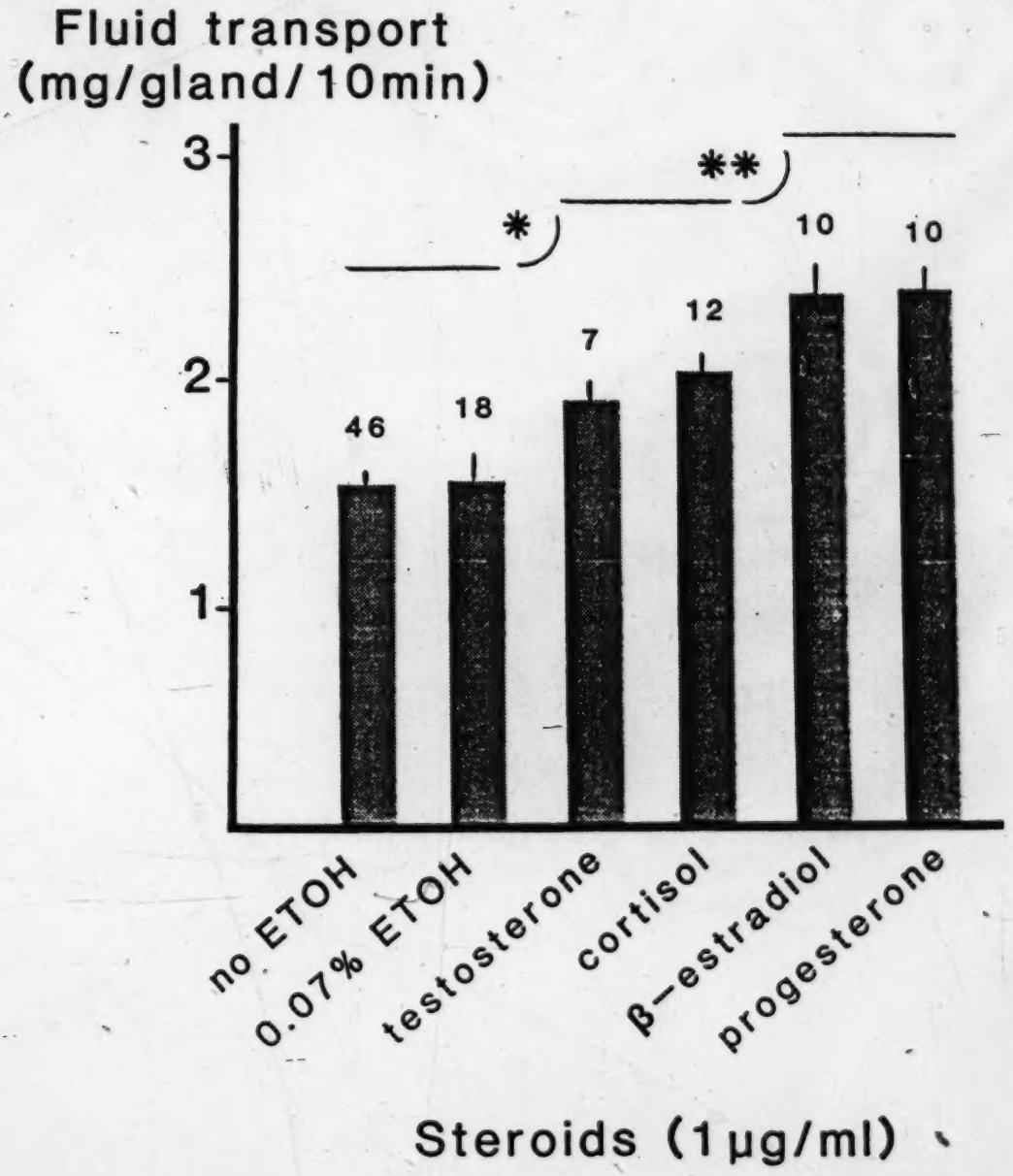

Fluid transport
(mg/gland/10min)
3
2
1
*
**
46
18
7
12
10
10
no ETOH
0.07% ETOH
testosterone
cortisol
β-estradiol
progesterone
Steroids (1µg/ml)

Fig. 11: Transmission electron micrographs of *A. americanum* salivary glands (type III acinus) cultured for 4 days in: A) 0.07% ETOH (mag. x15,000) or, B) 1 µg/ml 20-OHE (mag. x10,000). Note the abundance of autophagic vacuoles in the cells of the 20-OHE-treated salivary glands (arrows). M-mitochondria, Ml-membranous labyrinth.

A

B

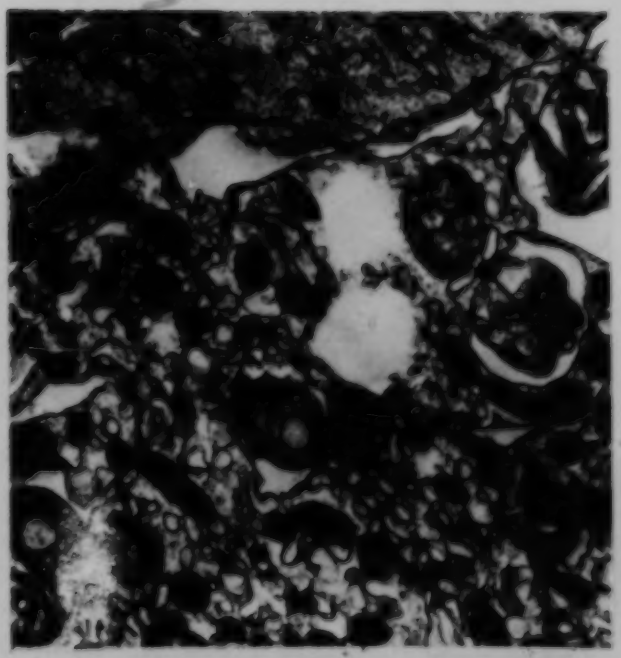
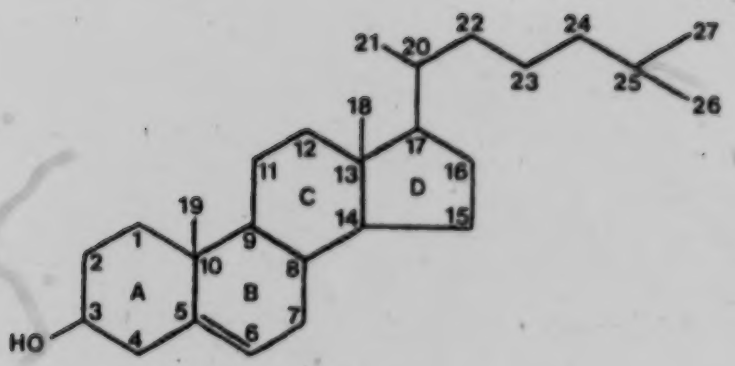


Fig. 12: Chemical structure (including ring and C-atom nomenclature) of cholesterol (taken from Lehninger, 1975).


HO
A
B
C
D
1
2
3
4
5
6
7
8
9
10
11
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13
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17
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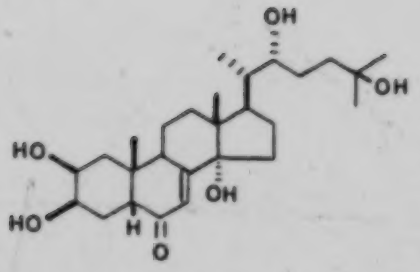
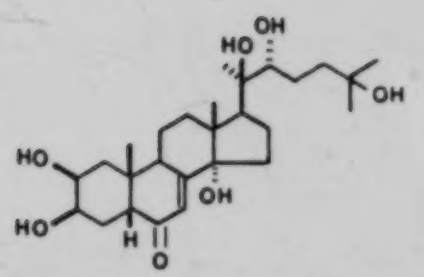
26
27

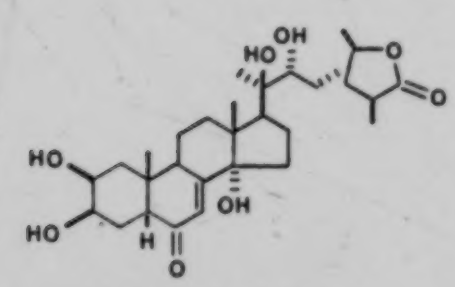
Fig. 13: Chemical structures of various ecdysteroids. All have a C-27 skeleton with a 6-keto-7-ene grouping in the B ring and, with the exception of 2-deoxyecdysone, a *cis*-fused A/B ring (taken from Karlson, 1980).



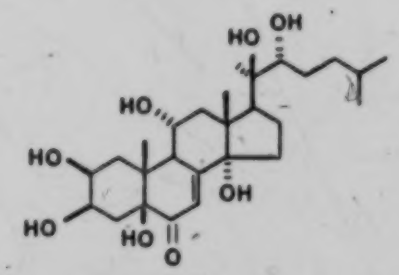
ecdysone



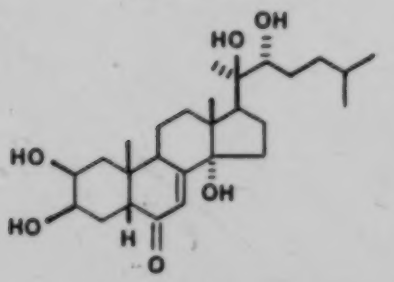
20-hydroxyecdysone



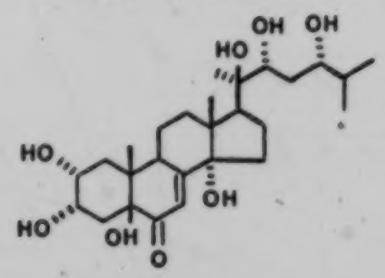
cyasterone



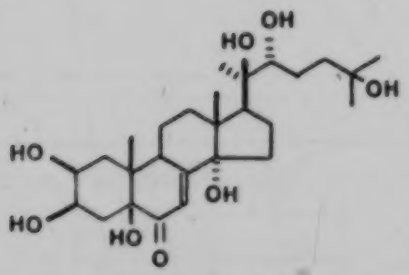
muristerone A



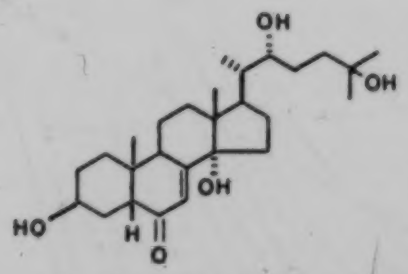
ponasterone A



ponasterone C

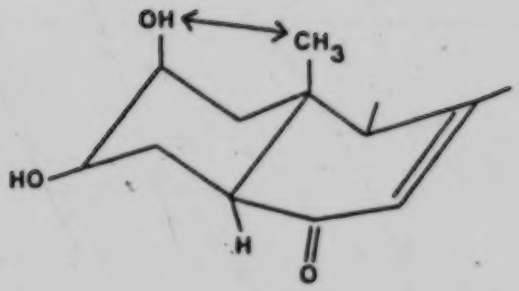


polypodine B

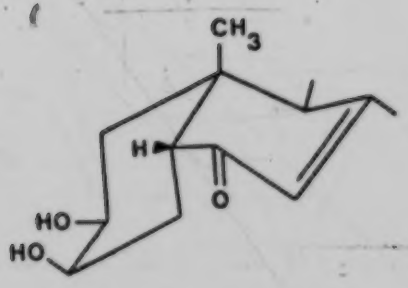


2-deoxyecdysone

Fig. 14: Diagrammatic representation of two isomers of the A/B ring of ecdysteroids: 5$\alpha$ and 5$\beta$. The 5$\alpha$ configuration is less stable due to the 2-$\beta$-OH and 19-$CH_3$ group interactions. As a result, active ecdysteroids have the 5$\beta$ configuration for the A/B ring (from Horn & Bergamasco, 1985).

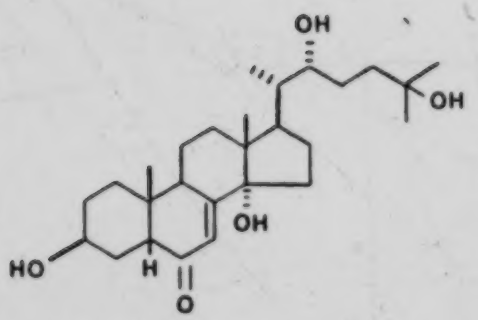



5α

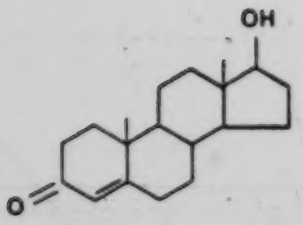



5β

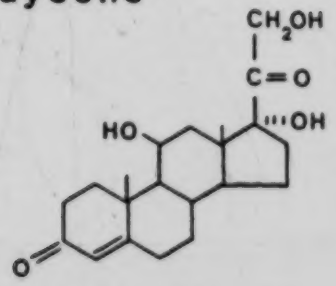
Fig. 15: Chemical structures of 2-deoxyecdysone and various vertebrate steroids (taken from Hadley, 1984). Due to the absence of the 2$\beta$-OH group in 2-deoxyecdysone, this ecdysteroid adopts a similar A/B ring conformation to the vertebrate steroids. All these steroids have a *trans*-fused A/B ring.



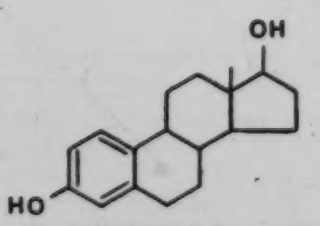

2-deoxyecdysone



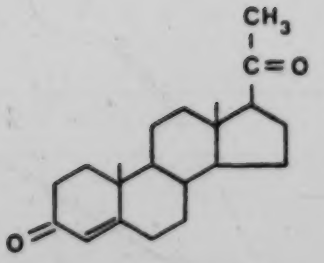

testosterone




cortisol




estradiol




progesterone

Fig. 16: Postulated (and generally accepted) model for the mechanism of action of steroid hormones. (R) - receptor, (H) - steroid hormone . See text for a description (modified from Hadley, 1984).

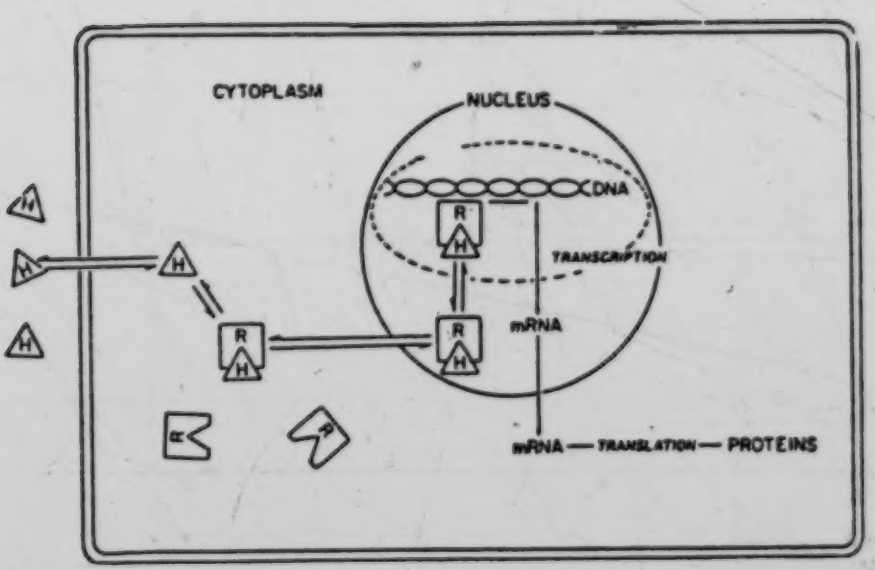
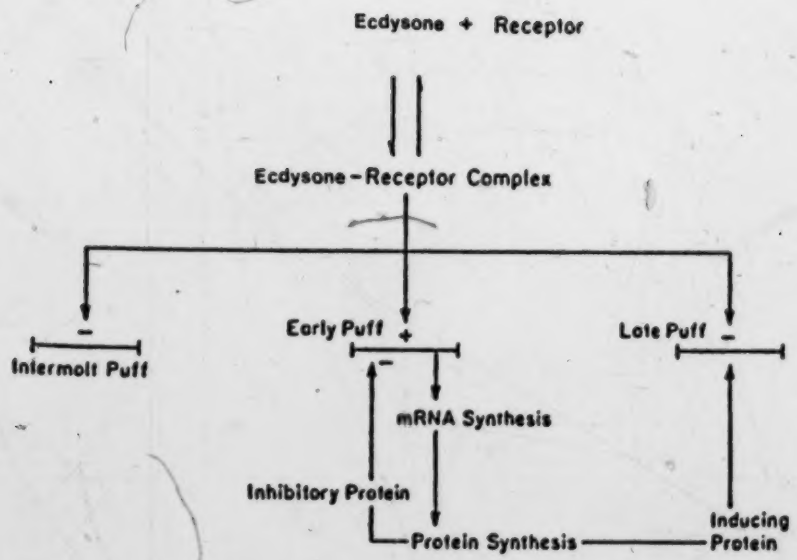

CYTOPLASM
NUCLEUS
DNA
TRANSCRIPTION
mRNA
mRNA — TRANSLATION — PROTEINS

Fig. 17: Model of the mode of action for ecdysteroid hormones. Consult the text for details (modified from O'Connor, 1985).


Ecdysone + Receptor
Ecdysone-Receptor Complex
Intermolt Puff
Early Puff
Late Puff
mRNA Synthesis
Inhibitory Protein
Protein Synthesis
Inducing Protein

## IV. The Effect of Azadirachtin on Salivary Gland Degeneration and Vitellogenesis

### A. Introduction

Ecdysteroids cause degeneration of salivary glands in the ticks, *A. hebraeum* (Harris & Kaufman, 1985) and *A. americanum* (this study). My *in vitro* studies suggest that E does not stimulate vitellogenesis in ticks. However, Connat *et al.* (1985) observed a correlation between the rise in haemolymph ecdysteroid titres and an increase in vitellogenesis in the ovary of *A. hebraeum*. It is still not established whether ecdysteroids stimulate vitellogenesis in ticks, or simply accumulate in the ovary as is the case for some insects.

It is now well established that E and 20-OHE regulate vitellogenesis in some insects. In most insects (eg. Orthoptera, Lepidoptera and Coleoptera) vitellogenesis is controlled predominantly by juvenile hormone (JH, see review by Koeppe *et al.*, 1985). However, E does play a role in some of these and other insects, and is the predominant hormone controlling vitellogenesis in higher flies (Hagedorn, 1985). However, E-induced vitellogenesis will not proceed in the ovary of these insects unless the ovary has been pre-exposed to JH (Flanagan & Hagedorn, 1977; Adams *et al.*, 1981).

If ecdysteroids control salivary gland degeneration and vitellogenesis in ticks, then an ecdysteroid inhibitor should block these processes. Azadirachtin is a potent insect growth regulator isolated from the neem tree, *Azadirachtica indica* (Juss). The general structure of azadirachtin is similar to E (fig. 18). At least one of the actions of azadirachtin is to inhibit the synthesis of E (Sieber & Rembold, 1983), or at least block its release (Rembold, 1984).

To further examine the role of ecdysteroids in ticks, I tested the effects of azadirachtin on two putative E-sensitive systems in the tick: salivary gland degeneration and vitellogenesis.

## B. Materials and Methods

### 1) Assay for Vitellogenesis

Tick vitellin is reddish brown due to its haem moiety. Thus, we could use the following spectrophotometric assay to measure the degree of vitellogenesis quantitatively:

Tissue preparation - the ovary was removed from the tick, blotted and weighed, homogenized in 3.5 ml of distilled water and stored in capped 4 ml disposable vials at -15°C. On the day of assay, the homogenates were thawed in a 25°C water bath and vortexed. The homogenate was pipetted into a 4 ml polycarbonate centrifuge tube and centrifuged at 4-6°C for 15 min at 20,000xg in an International IEC centrifuge (model M-25). The supernatant was collected.

Spectrophotometry - after centrifugation, the supernatant often remained slightly cloudy. As this would increase the baseline absorbance in proportion to the original amount of tissue, absorbance at 500 nm (non-specific for vitellin) was subtracted from absorbance at 400 nm (near the peak for the haem moiety of vitellin) using a Beckman DU-8 Spectrophotometer. The corrected absorbance was then normalized for ovary weight. Homogenates of eggs served as a standard for maximal possible vitellogenesis.

### 2) Injections

In one experiment, ticks received 50 µg azadirachtin/g tick body weight (bw). Concentrated stock solutions of

azadirachtin (0.05 mg/µl; a gift from H. Rembold, Martinsreid, F.G.R. and J. Koolman, Marburg, F.G.R.) were dissolved in 60% dimethylsulfoxide (DMSO; Sigma). The stock was diluted to 5 µg/µl with distilled water prior to injection, so that final DMSO concentration was 6%. When lower concentrations of azadirachtin were injected (1-25 µg/g bw), dilutions were made such that the final DMSO concentration was also adjusted to 6%. Control ticks were injected with 6% DMSO. Injections were made using an 'Agla' micrometer syringe (Wellcome Reagents, Ltd). These 1 ml all-glass syringes were fitted with a 30 gauge needle which was inserted in the tick through the camerostomal fold. On pushing the tick gently forward the needle tip entered a few mm into the haemocoel without rupturing the gut diverticula. In all cases, the injected volume was 1 µl/100 mg bw.

**3) Experiments**

Experiment 1: To determine the critical weight above which vitellogenesis begins, partially fed females (20-170 mg) were removed from the host and their ovaries were excised at day 0,4 or 7 post-removal. Spectrophotometric analysis was performed on the excised ovarian homogenates (fig. 19).

Experiment 2: To examine the normal course of vitellogenesis, engorged females were removed from the host and their ovaries were excised at days 0-11 post-engorgement

(fig. 20). These ticks were held at 26°C, 95% RH in the interim.

Experiment 3: Azadirachtin was tested in 2 ways; by single or double injection. Single injections: In a preliminary experiment, females were collected on the day of engorgement and injected with 25 µg/g azadirachtin. Double injections: Small partially fed ticks were removed from the host, injected with 0,1,3,10 or 50 µg/g azadirachtin, painted with coloured nail polish to identify the azadirachtin concentration, and returned to the host. When these females had engorged (1 to 3 days later), they were injected a second time with the same concentration of azadirachtin. When oviposition began (single and double injected ticks), eggs were collected and weighed every third day for 9 days. One and a half months later, remaining eggs were collected and weighed (figs. 21 & 22).

For the following reasons, egg weight is not of itself an appropriate unit to compare egg masses. It has been shown (Kaufman *et al.*, 1986), that the injection method used here often damages the egg-waxing organ of ticks (Gené's organ). If damage is severe, the laid eggs quickly dry out and have a much darker colour than normal eggs. When compared with normal eggs in the spectrophotometer, this would lead to abnormally high readings per unit egg mass. Also, the dried eggs obviously weigh less than normal eggs, so recording only egg weights would give misleadingly low values for egg production. Therefore, small batches of eggs were collected

from a series of untreated females and from injected females. The small batches were weighed and the eggs counted in each batch. Egg number/mg egg weight was calculated for each female, standardized for each tick and, therefore, recorded as egg number/g bw.

Experiment 4: The same basic protocol for double injections in experiment 3 was adopted for this experiment. However, instead of allowing oviposition to occur, the ovaries were removed 7 days post second injection, homogenized and assayed for degree of vitellogenesis (fig. 23). The salivary glands were also removed and tested for secretory competence.

C. Results

Experiment 1: Ovaries excised from all ticks at day 0 post-removal showed no signs of vitellogenesis (fig. 19). Similarly ovaries removed from small partially fed females had not accumulated vitellin by day 4 or 7. The appearance of vitellin in the ovaries was apparent only in day 4 post-removal ticks weighing >60 mg.

Experiment 2: The normal course of vitellogenesis for engorged female ticks is shown in fig. 20. Vitellogenesis reached its peak by day 4 and remained at that level throughout the experiment even after oviposition began. Engorged *A. americanum* usually begin oviposition about 7 days post-engorgement (pers. observation).

Experiment 3: A single injection of 6% DMSO into engorged ticks had no significant effect on egg production compared to untreated ticks: untreated females laid 9015 ± 544.6 eggs/g bw (n=12), whereas singly-injected ticks laid 8329 ± 953.2 eggs/g bw (n=11, $p>0.05$). A single injection of 25 µg/g azadirachtin also did not affect total egg production. A double injection of azadirachtin was equally ineffective on total egg production (fig. 21). Although there was a slight reduction in egg numbers laid by twice injected ticks (10 and 50 µg/g azadirachtin, fig. 21), this decline was not statistically significant (8102 ± 663.4 eggs/g bw, n=C for controls vs.

7054 ± 348.3 eggs/g bw, n=21 for 10+50 µg/g; $p>0.05$). However, high concentrations of azadirachtin (10 and 50 µg/g) did lower somewhat the rate of eggs produced for the first 6 days compared to eggs laid by ticks injected with 1 µg/g ($p<0.01$, fig. 22).

Azadirachtin also delayed the onset of oviposition. Untreated ticks oviposit 7.1 ± 0.3 days (n=12) post engorgement. A double injection of 6% DMSO did not significantly retard oviposition (10.0 ± 1.2 days (n=8), $p>0.05$ compared to untreated controls). Ticks injected with 10 µg/g azadirachtin began to oviposit 13.3 ± 2.5 days ($n=10$, $0.01<p<0.05$) post-second injection. Although azadirachtin delayed oviposition, it was not dose dependent.

As mentioned earlier, the site of injection destroyed Gené's organ; thus, eggs laid by injected females dried out and weighed less. Consequently, one would expect there to be more eggs/mg laid from injected ticks. Indeed, this was the case: untreated females oviposit 17.7 ± 0.3 eggs/mg (n=5), whereas twice injected ticks laid on average 37.5 ± 1.3 eggs/mg (n=18).

Experiment 4: Azadirachtin, twice-injected up to 50 µg/g caused no significant reduction in vitellogenesis after 7 days (fig. 23). Also, azadirachtin (1-50 µg/g) did not prevent salivary gland degeneration in any of the females tested (data not shown).

**D. Discussion**

As much as 50 µg/g azadirachtin was ineffective in blocking salivary gland degeneration and vitellogenesis in *A. americanum*. A number of studies on insects suggest that a single injection of azadirachtin at a dose of 0.1-10 µg/g is usually effective (Sieber & Rembold, 1983; Ladd *et al.*, 1984; Schlüter, 1985; Barnby & Klocke, 1987). A number of possibilities could explain the lack of effect in ticks. 1) There may have been enough endogenous E in >300 mg ticks such that a single injection of azadirachtin had been administered too late to block E's effect. Harris & Kaufman (1985) have shown that a critical time exists for TSGDF release. Salivary glands of large *A. hebraeum* ticks put into organ culture less than 24 h post-removal do not degenerate 4 days later. When similar ticks are set up in culture after 24 h post-removal from the host, their salivary glands degenerate. This suggests that once TSGDF is released and enters the cell (i.e. within 24 h post-removal), salivary gland degeneration can not be blocked by washing away TSGDF. Therefore, I injected some ticks before the critical weight (<60 mg) and put them back on the host to feed again. After repletion, azadirachtin was re-injected. This ensured that the original dose would not be too dilute to inhibit subsequent ecdysteroid synthesis. Figs. 21-23 indicate that azadirachtin was still ineffective.

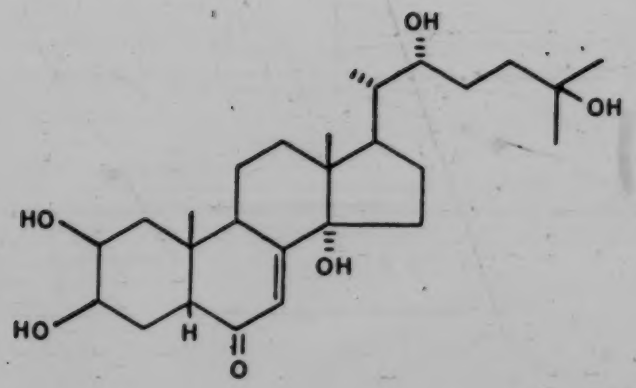
2) Some insects are quite resistant to azadirachtin. For example, Japanese beetle pupae older than 72 h are not affected by 0.1-0.4 µg azadirachtin even though larval and <72 h pupal stages are affected (Ladd *et al.*, 1984). Perhaps *A. americanum* also has such an azadirachtin-resistant ecdysteroid system.

3) Ecdysteroids may not be involved in vitellogenesis in *A. americanum*. I mention once again that no one has yet been able to demonstrate a direct effect of ecdysteroids on vitellogenesis in ixodid ticks. Connat *et al.* (1985) only showed a correlation between increasing ecdysteroid titres in the haemolymph and degree of vitellogenesis, as well as ovarian uptake of E. Possibly 20-OHE is eliciting its action elsewhere. For example, in many insects, rising ovarian ecdysteroid levels occur as the eggs mature. This reflects the ecdysteroids that are sequestered by the egg and probably used in embryogenesis. Lagueux *et al.* (1981) using RIA/HPLC on embryos of *Locusta migratoria*, found high levels of E and 2-deoxyecdysone in free and conjugated (stored) forms. The embryos apparently use the ecdysteroid for cuticle deposition (Lagueux *et al.*, 1979). Similarly, [$^3$H]-20-OHE injected into fed *O. moubata* ticks is sequestered by the ovary during vitellogenesis, metabolized and then incorporated into the eggs (Connat *et al.*, 1984). In these arthropods, ecdysteroids apparently do not promote nor regulate vitellogenesis.

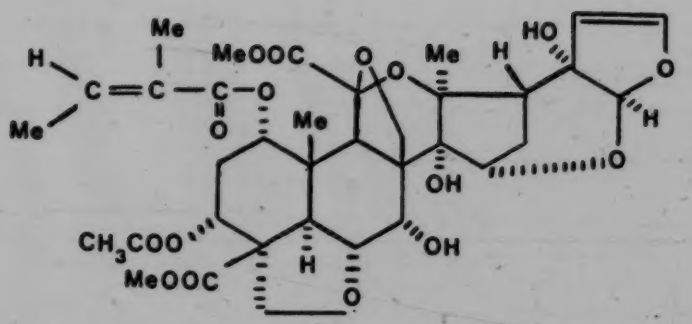
In contrast to the above, vitellogenesis in argasid ticks is inhibited by ecdysteroids. Ecdysteroids reportedly cause complete egg resorption in the ovaries of *Ornithodoros moubata* (Connat & Diehl, 1986). Large doses of ecdysteroids reduce fecundity (Connat *et al.*, 1986) and JH supposedly elicits egg maturation and oviposition in the same tick (Connat & Diehl, 1986). Pound & Oliver (1979) demonstrated that precocene (a JH antagonist) prevents oogenesis in *O. parkeri* and that a subsequent application of JH leads to partial recovery.

Although dispute exists over E involvement in tick vitellogenesis, evidence for its mediation in salivary gland degeneration is now quite strong, yet azadirachtin has no effect. Once again, possibly azadirachtin does not affect all ecdysteroid systems. Before one can say more, however, the cellular mechanism whereby azadirachtin exerts its effect in sensitive insects has to be established. Contrary to my initial hopes, the tick salivary gland system is not a good model to explore this.

Fig. 18: Chemical structure of: (A) - ecdysone (Karlson, 1980) and, (B) - azadirachtin (Warthen, 1979).


OH
OH
HO
OH
HO
H
O

A


Me
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C
C
O
Me
O
MeOOC
O
O
Me
H
HO
O
H
O
Me
OH
CH3COO
OH
H
MeOOC
O

B

Fig. 19: Absorbance of ovaries from partially fed *A. americanum* ticks (20-170 mg) on day 0 (◯), day 4 (△) and day 7 (□) post-removal. Incorporation of vitellin into the ovary occurs only in ticks >60 mg. Means ± S.E.M., n are shown.

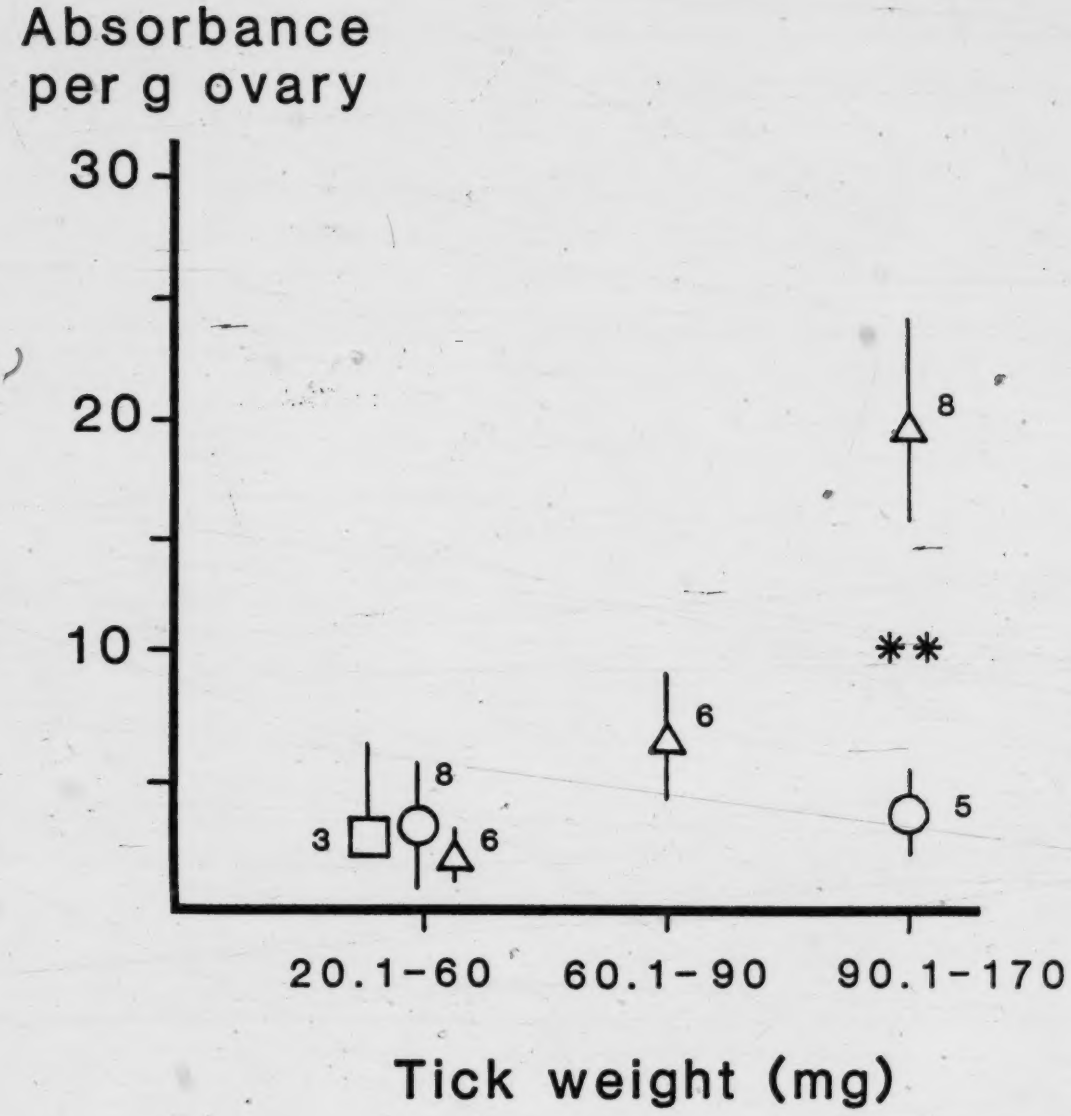

Absorbance
per g ovary
30
20
10
8
**
6
8
3
6
5
20.1-60
60.1-90
90.1-170
Tick weight (mg)

Fig. 20: Absorbance of ovarian homogenates from replete *A. americanum* ticks (>500 mg) as a function of time post-engorgement. Eggs laid were also analyzed to show the maximum absorbance possible (◇). See text for experimental protocol. Means ± S.E.M., n are shown. Vitellogenesis increased significantly ($0.01<p<0.05$) and reached a plateau on day 4, at which point vitellogenesis remained constant even after ovipostion began (day 6-7). Only day 8 ovaries deviated significantly from the plateau ($p<0.01$ compared to day 7; $p>0.05$ compared to day 9).

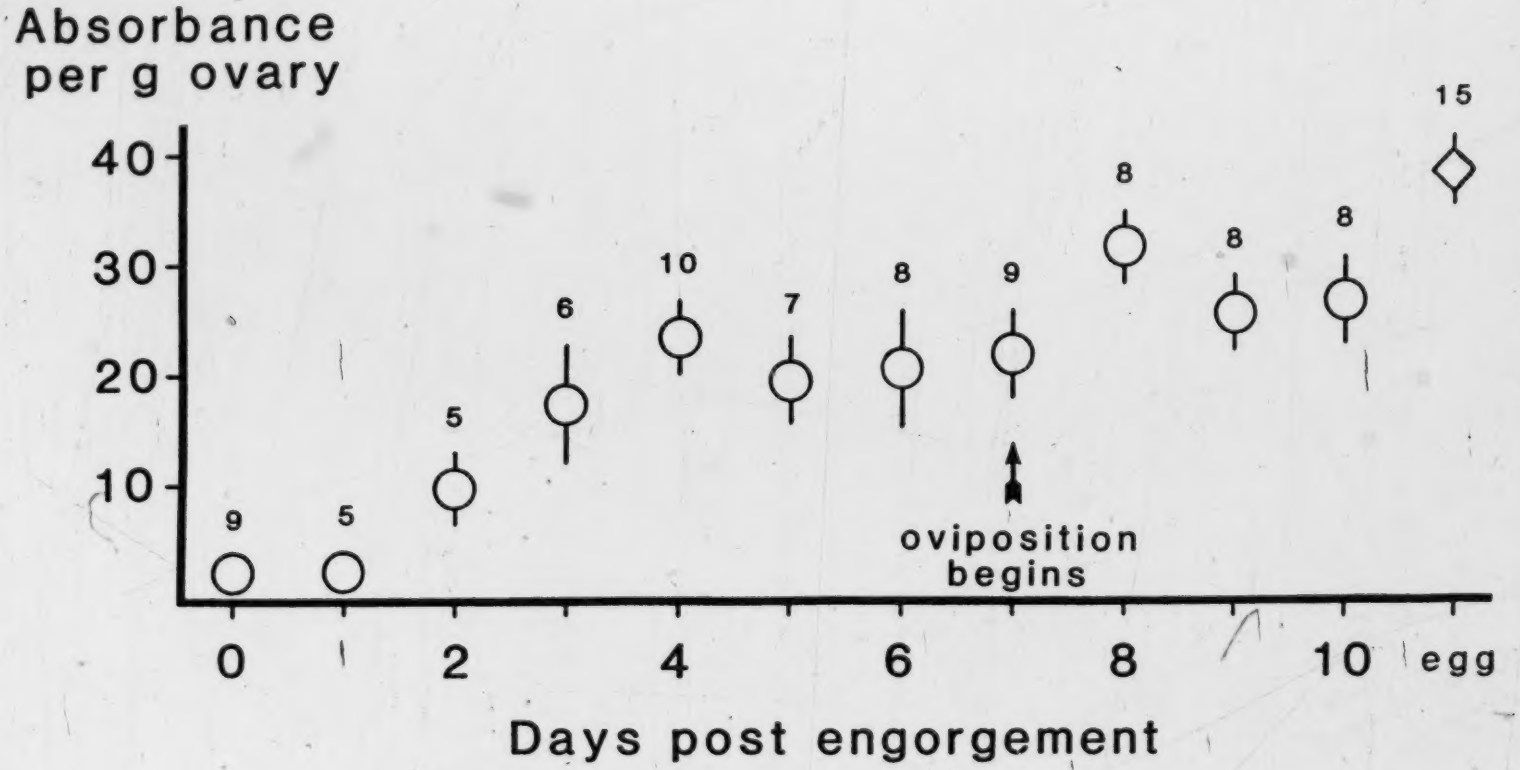

Absorbance
per g ovary
40
30
20
10
9
5
5
6
10
7
8
9
8
8
8
15
oviposition
begins
0
2
4
6
8
10
egg
Days post engorgement

Fig. 21: Effect of azadirachtin on egg production in large *A. americanum* ticks (>300 mg). Small partially fed ticks were removed from the host and injected with a concentration of azadirachtin (1 µl/100 mg body weight). They reattached to the host and fed to repletion. These now engorged ticks were injected a second time with the same azadirachtin concentration. The ticks were then put into an incubator and allowed to oviposit. Total egg number laid was recorded for each tick. Azadirachtin had no significant effect on total egg production ($p>0.05$). Means ± S.E.M. and n are shown.

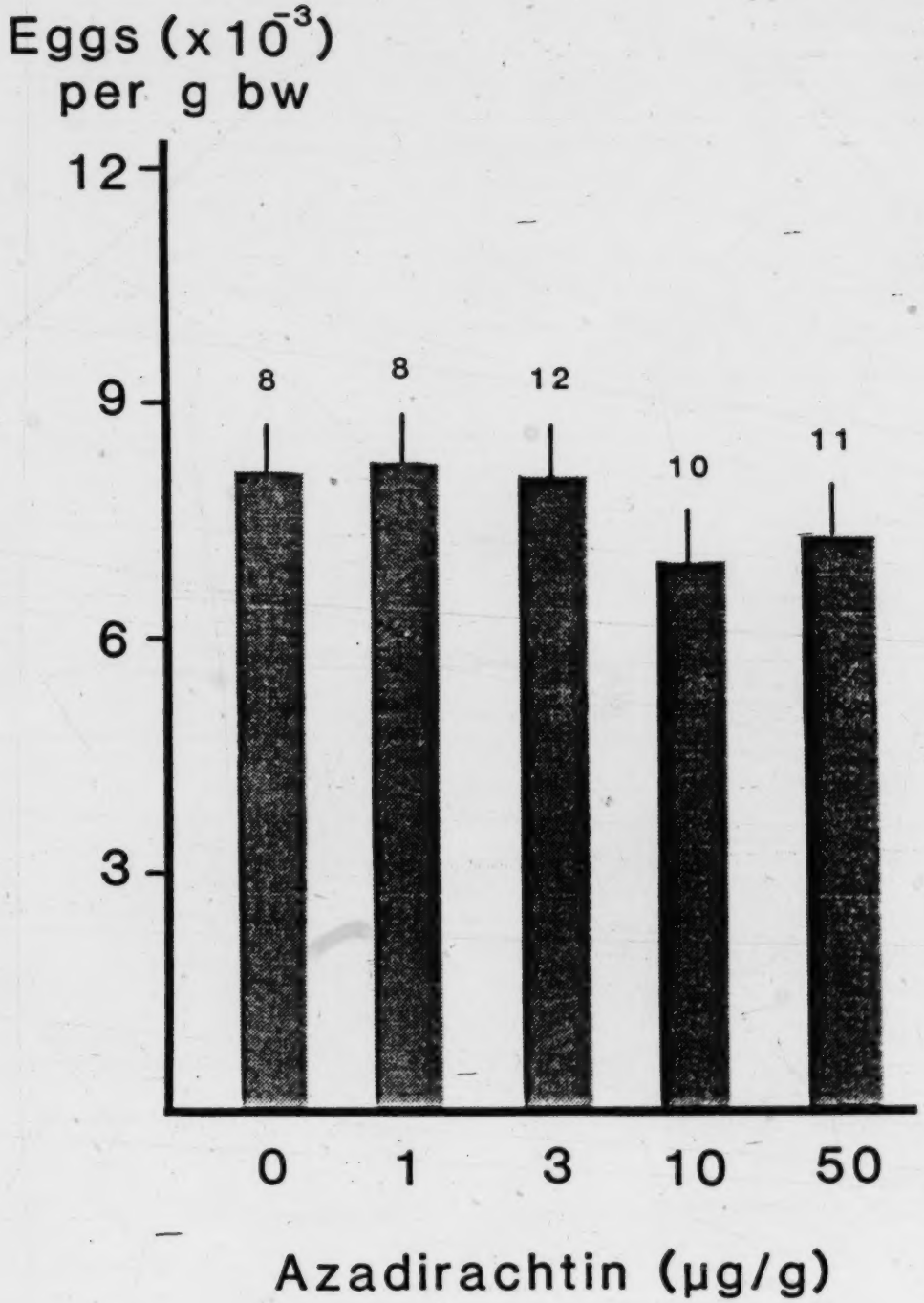

Eggs (x 10⁻³) per g bw
12
9
6
3
8
8
12
10
11
0
1
3
10
50
Azadirachtin (µg/g)

Fig. 22: Effect of azadirachtin on the rate of oviposition in large *A. americanum* ticks (>300 mg), treated as described in fig. 21. Azadirachtin concentrations were (µg/g): (◯) control (6% DMSO), (◇) 1, (□) 3, (△) 10, (◇) 50. Means ± S.E.M., and n are shown. By day 3, 10 and 50 µg/g azadirachtin-injected ticks laid fewer eggs than 1 or 3 µg/g azadirachtin-injected ticks ($p<0.01$). By day 6, 10 and 50 µg/g azadirachtin-injected ticks were laying fewer eggs than 1 µg/g azadirachtin-injected ticks ($0.01<p<0.05$) but not control ticks ($p<0.08$). Egg batches for each 3 day period are not cumulative.

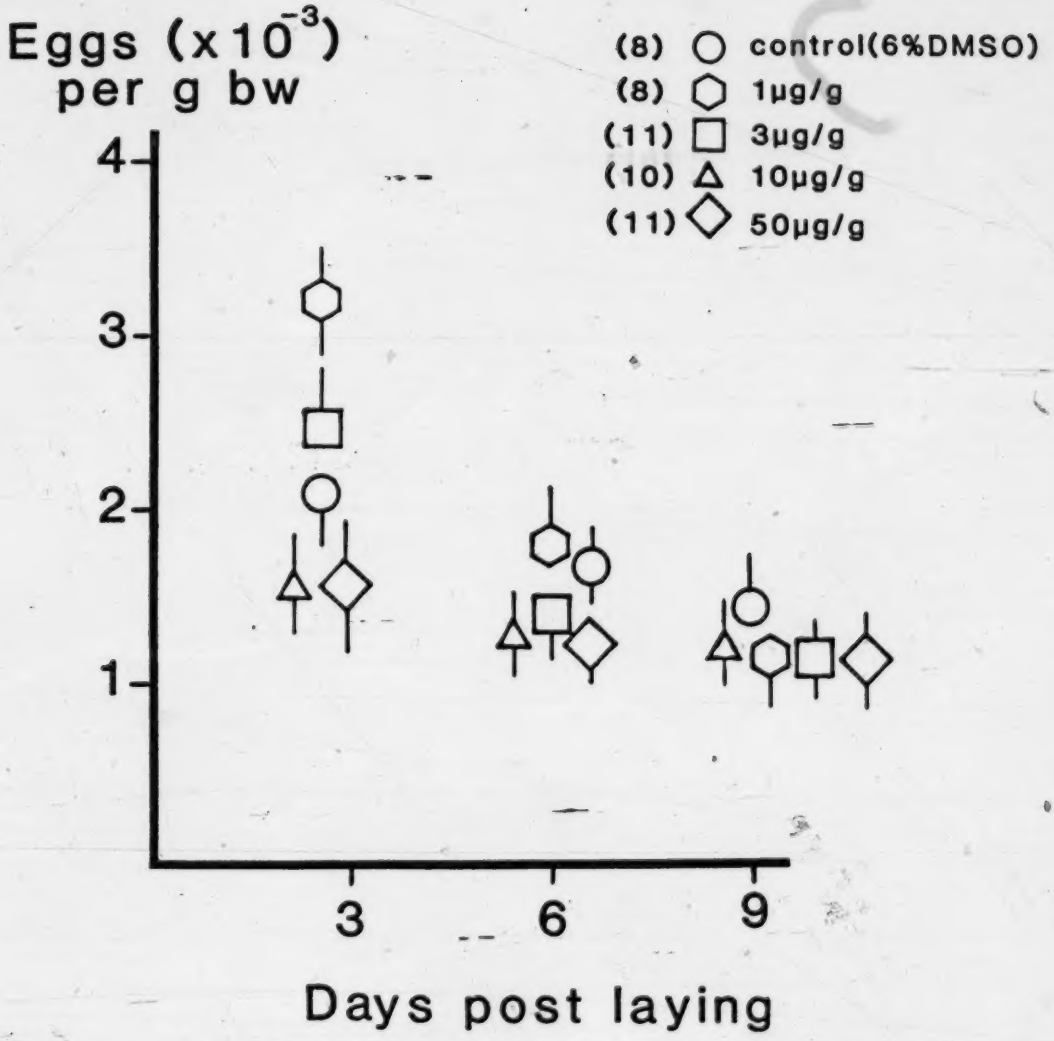
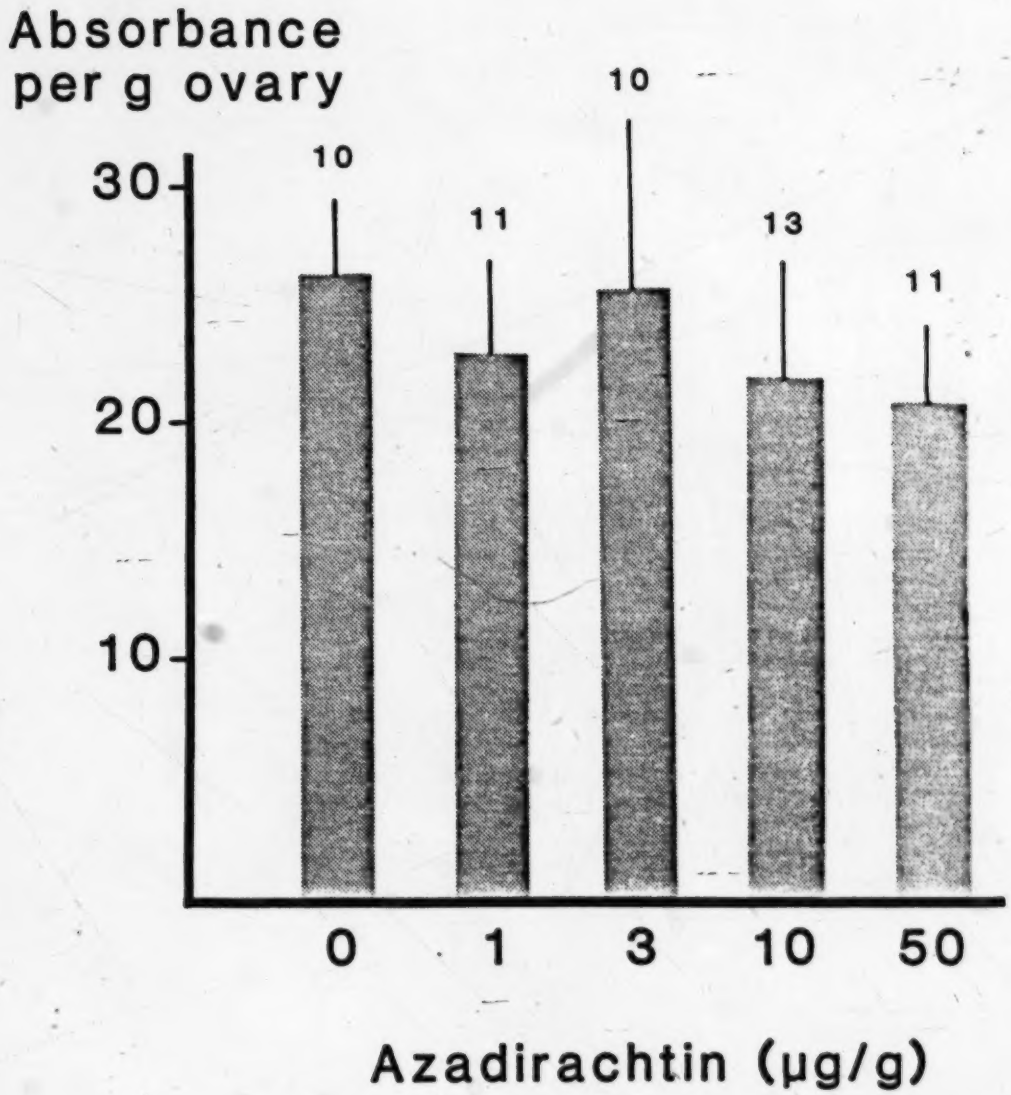

Eggs (x10$^{-3}$) per g bw
4
3
2
1
3
6
9
Days post laying
(8) control(6%DMSO)
(8) 1µg/g
(11) 3µg/g
(10) 10µg/g
(11) 50µg/g

Fig. 23: Effect of azadirachtin on vitellogenesis in large *A. americanum* ticks (>300 mg). Small partially fed ticks were removed from the host and injected as described in fig. 21. Seven days post-second injection, ovaries were excised and assayed. Means ± S.E.M. and n are shown. Azadirachtin had no significant effect on vitellogenesis ($p>0.05$).


Absorbance
per g ovary
30
20
10
10
11
10
13
11
0
1
3
10
50
Azadirachtin (µg/g)

# V. General Conclusion

## A. Salivary Gland Degeneration

Degeneration of the salivary glands is triggered by TSGDF. In *A. americanum*, autolysis begins at a fed weight of 60-70 mg compared to 250-350 mg for A. hebraeum (Harris & Kaufman, 1984). Weights of unfed individuals of the two species are 3-5 mg and 20-35 mg, respectively. Thus, in two species of ixodid ticks, the critical weight above which TSGDF is released is approximately 10 times the unfed weight. Therefore, it is quite possible that stretch receptors in the abdomen may be one component responsible for controlling TSGDF synthesis/release.

TSGDF is probably an ecdysteroid. Ecdysteroids have been found in several ticks (see Dees *et al.*, 1985), yet their action in ticks has not been fully studied. I have shown that ecdysteroids at physiological concentrations cause the salivary glands to degenerate in *A. americanum*. Indeed, 20-OHE in organ culture promoted autophagic vacuole activity within the type III acini. Other steroids (progesterone, testosterone, $\beta$-estradiol and cortisol) increased fluid secretory competence after 4 days in organ culture. Thus, the effect on degeneration is specific to the ecdysteroid family of steroids.

Ecdysteroid activity in insects depends on certain structural requirements of the molecule. Specifically, active ecdysteroids must contain: a) a *cis*-fused A/B ring, b) a 6-keto-7-ene grouping in the B ring, and c) a full sterol side chain (Horn & Bergamasco, 1985). The vertebrate steroids and 2-deoxyecdysone have a *trans*-fused A/B ring and the vertebrate steroids lack the other two features indicated. Why the vertebrate steroids and 2-deoxyecdysone improve fluid secretion is not known. The glucocorticoid lysosomal stabilization hypothesis (Zurier & Weissmann, 1973) has been suggested but it is not without its weaknesses.

**B. Vitellogenesis**

I could not demonstrate that ecdysteroids trigger vitellogenesis *in vitro*, but the *in vitro* preparation required removal of haem compounds, guts and much of the fat body. The guts and fat body both synthesize vitellin in ticks (Coons *et al.*, 1986). As a result, substrates and/or machinery necessary for vitellin synthesis may have been absent. Thus, the ecdysteroids may have been ineffective for the latter reasons only. I did not test a direct effect of ecdysteroids on vitellogenesis *in vivo*; however, azadirachtin (an ecdysteroid synthesis inhibitor, Rembold & Sieber, 1983), did not block vitellogenesis nor reduce

fecundity in engorged *A. americanum*. Surprisingly, azadirachtin also did not attenuate salivary gland degeneration *in vivo*, a system which is ecdysteroid-sensitive. Therefore, at least one ecdysteroid system (salivary gland) is insensitive to azadirachtin. The lack of effect of azadirachtin on vitellogenesis may be due to one of two reasons: 1) Ecydsteroids do not stimulate vitellogenesis in ticks. Indeed, no one has yet demonstrated such a direct effect. 2) Like the salivary glands, vitellogenesis is an azadirachtin-insensitive system.

**C. Suggested Experiments**

Autolysis of salivary gland tissue occurs above the critical weight of 60-70 mg in *A. americanum*. Above the critical weight, does a correlation exist between haemolymph ecdysteroid titres and salivary gland degeneration? RIA/HPLC studies may indicate whether a surge in ecdysteroid titre occurs once the tick feeds beyond the critical weight.

As previously mentioned, stretch in the abdomen may trigger TSGDF release. One possible location of these putative stretch receptors could be the dorso-ventral musculature. Harris & Kaufman (1984) showed that severing the opisthosomal nerves (those which innervate the musculature) inhibits salivary gland degeneration. Experiments should be conducted to examine what effect

opisthosomal nerve cutting has on ecdysteroid titres in the haemolymph, ovary and synganglion.

The control of vitellogenesis in ticks is still uncertain. My *in vitro* preparation would be useful for examining vitellogenesis providing the machinery required (guts, fat body, haem compounds) are intact. This is feasible to do with my culture method. Vitellogenesis in argasid ticks appears to be regulated by JH and its analogues (see chapter 4). The organ culture method may prove useful also to study JH effects on vitellogenesis in ixodid ticks.

Although ecdysteroids have been found in a variety of tick species, the ecdysteroid receptor in ticks is still unidentified. Due to the relative ease with which the salivary gland can be isolated and its obvious sensitivity to ecdysteroids, it would be a good system in which to identify the putative ecdysteroid receptor.

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## Appendix

## Composition and Preparation of Solutions

1) HANK'S MEDIUM

| Compound | mg/l |
|---|---|
| $CaCl_2$ | 140.0 |
| D-glucose | 1600.0 |
| KCl | 400.0 |
| $KH_2PO_4$ | 60.0 |
| $MgSO_4$ | 98.0 |
| NaCl | 11 500.0 |
| $NaHPO_4$ | 47.5 |
| Phenol Red | 10.0 |

Dissolve all ingredients in somewhat less than total volume omitting the $CaCl_2$. Dissolve the $CaCl_2$ in a small volume of $H_2O$, and add slowly to the remaining solution, stirring constantly. Adjust pH to 7.2 and q.s. with $H_2O$ to 1 litre. Bring solution to room temperature before use.

2) TC MEDIUM 199

1 package TC 199 (Gibco Chemical Co., Cat. #400-1200)
2.1 g MOPS (3-[n-Morpholino] propanesulfonic acid; Sigma)
2.09 g NaCl

Mix ingredients in enough $H_2O$ to bring to 1 litre. Adjust pH to 7.2 and bring to room temperature before use.

3) STERILE TC MEDIUM 199

Prepare TC medium 199 as previously described (1 litre). Add 5 ml Gentamicin sulfate (10 mg/ml stock; Sigma) such that final Gentamicin concentration is 50 µg/ml in 1 litre. Filter solution through a sterile millipore apparatus attached to a vacuum.

4) MELLONIG'S PHOSPHATE BUFFER (from Mellonig, 1961)

| Compound | g/l |
|---|---|
| $NaH_2PO_4.2H_2O$ | 5.85 |
| $Na_2HPO_4$ | 15.25 |

Dissolve ingredients in 1 litre distilled $H_2O$.